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# Mild hypercapnia before reperfusion reduces ischemia-reperfusion injury in hyperacute ischemic stroke rat model

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## Abstract

Endovascular thrombectomy has a recanalization rate over 80%; however, approximately 50% of ischemic stroke patients still experience dependency or mortality. Recently, clinical trials demonstrated the benefits of administering neuroprotective agents prior to endovascular thrombectomy. Additionally, recent studies showed neuroprotective effects of mild hypercapnia in patients resuscitated after cardiac arrest. However, its efficacy in ischemic stroke remains unclear. We aimed to investigate whether carbon dioxide ($CO_2$) per-conditioning has neuroprotective effects in rat models with middle cerebral artery occlusion (MCAO). Rat models received intermittent inhalation of mixed gas during the MCAO period. After surgery, behavioral assessments, infarct size measurement, immunohistochemistry, and western blot analysis were performed. We found $CO_2$ per-conditioning reduced infarct size and neurological deficit. The number of 8-hydroxy-2-deoxyguanosine (8-OHdG) positive cells and matrix metalloproteinase 9 (MMP-9)/platelet derived growth factor receptor beta (PDGFRβ) double positive cells were significantly decreased after $CO_2$ per-conditioning. The expressions of tight junction protein and pericytes survival were preserved. This study underscores mild hypercapnia before reperfusion not only reduces neurologic deficit and infarct size, but also maintains the integrity of the blood-brain barrier and neurovascular unit, alongside mitigating oxidative stress in hyperacute stroke rat models. Therapeutic mild hypercapnia before reperfusion is promising and requires further clinical application.

## Keywords

Carbon dioxide, per-conditioning, oxidative stress, blood-brain barrier, ischemia-reperfusion injury

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## Introduction

Acute stroke is the second leading cause of morbidity and mortality worldwide.[1] A significant proportion of patients with 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.[2] Due to developments in mechanical devices and the technical skills of EVT, the recanalization rate has dramatically increased to approximately 80%.[3] However, the rate of favorable outcomes remains at approximately 50% despite proper recanalization with timely treatment.[3]

Most of the neuroprotective trials are aimed for pre-conditioning mechanism. It may not be applicable in clinical settings since treating patients before an

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occurrence of acute ischemic stroke is nearly impossible, limiting its real-world relevance.[4] In contrast, per-conditioning may be more suitable because per-conditioning is carried out between onset of ischemia and reperfusion by EVT. Clinically, per-conditioning corresponds to a treatment just before EVT to prevent ischemia-reperfusion injury. A recent phase III clinical trial (ESCAPE-NA1) found that administering nerinetide prior to EVT improved functional outcomes and lowered mortality in patients who did not receive alteplase.[5]

Carbon dioxide ($CO_2$) is a harmless gas and has powerful vasodilative effects on the cerebral vessels.[6] $CO_2$ is maintained within narrow range (35–45 mmHg) in physiologic state and has an intense diffusion capacity with an ability to cross the blood-brain barrier (BBB). Several studies showed that arterial carbon dioxide tension ($PaCO_2$) is strong determinant of cerebral blood flow (CBF). Furthermore, $CO_2$ has anti-oxidative, anti-epileptic, and anti-inflammatory effects.[4,7–12] Two recent randomized clinical trials on mild hypercapnia in post-cardiac arrest patients yielded mixed results. One study observed benefits in comatose patients, while the other found no significant impact, despite differing primary endpoints.[13,14] To date, there have been no clinical trials that specifically investigate mild hypercapnia in patients with acute ischemic stroke.

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).[15] This contributes to ischemia-reperfusion injury, affecting the peri-lesional penumbra.[16] Ischemia-reperfusion injury has multiple mechanisms to make cell dysfunction like overproduction of ROS, consumption of antioxidants, inflammation, BBB disruption, apoptosis, and increasing excitotoxicity.[17,18] As a result, cerebral damage expands to encompass the penumbra and adjacent brain parenchyma.[19] Therefore, how to avoid or reduce ischemia-reperfusion injury is important, which is called neuroprotection.[19,20] Until recently, a number of neuroprotective agents were developed and subjected to clinical trials. However, despite their success in preclinical studies, only few agents have proven successful in clinical trials.[21,22]

During ischemia-reperfusion injury, pericytes in blood microvessels undergo significant changes.[23–25] In such events, pericytes tend to remain contracted, contributing to the no-reflow phenomenon.[26,27] Mitigating oxidative stress could potentially induce pericytes contraction and enhance their survival, thereby restoring microcirculatory flow.[26] Nonetheless, the impact of mild hypercapnia on pericytes survival remains unclear.

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, blood-brain barrier disruption, and pericytes survival.

## Materials and methods

### 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 experiments were conducted in compliance with the Animal Welfare Act, National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize suffer and the number of rats. In total, 42 male Wistar rat (8-week-old, 270–320 g) 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 $22°C \pm 2°C$. The results are presented according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) reporting guideline.

### Experimental design and protocol

Before the operation, rotarod training had been performed for three days. We made the middle cerebral artery (MCA) infarction models. 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. To explain briefly, the left internal carotid artery (ICA) was incised using micro-scissors to insert the monofilament (4035556PK10; Doccol Co., Albuquerque, NM, USA) and the filament was cautiously advanced up to 20 mm into the ICA to occlude the origin of the MCA. The MCA was occluded for 90 min. After 30 min from induction of the MCA occlusion (MCAO), the rat began inhaling a mixed gas (20% $CO_2$, 20% $O_2$, 60% $N_2$; flow rate = 1 L/min) with 3 cycles of inhalation for 5 min followed by 10 min recovery session (normoxic atmosphere). $CO_2$ inhalation was conducted through the self-respiration of animals in a sealed cage where mixed gas was balanced. 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 same surgical procedure without any occlusion, while the animals with permanent MCAO (pMCAO) did not undergo the microfilament removal procedure (Figure 1). The physiological variables were recorded by arterial blood gas analyses (ABGA) before

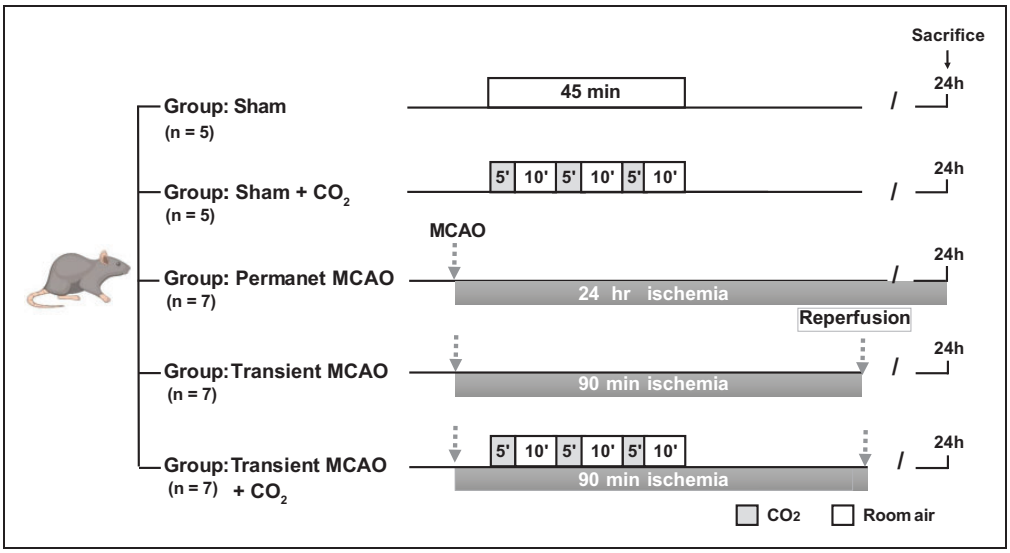


**Figure 1.** Experimental design. $CO_2$: carbon dioxide; MCAO: middle cerebral artery occlusion.

and after the $CO_2$ inhalation. All animals were sacrificed at 24 hours post-surgery, and the tMCAO groups had a reperfusion time of 22 hours and 30 minutes.

### *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 mean difference of infarct size between the tMCAO group and the tMCAO + $CO_2$ inhalation group was 18.15. By inputting the standard deviation for each group and calculating, the final optimal sample size was determined to be 4 animals per group. Similarly, the mean difference between the pMCAO group and the tMCAO + $CO_2$ inhalation group was 21.37. After inputting the standard deviation for each group and calculating, the final sample size was also determined to be 4 animals per group. To account for an anticipated mortality rate and technical errors of 40%, the study included 42 rats in total.

To verify the presence of salvageable tissue in the tMCAO models, pMCAO models were included in the study groups. A total of 42 rats practiced rotarod training, but 11 rats failed the training. Therefore, 31 rats were randomly allocated into five groups (Supplementary Figure 1). sham-operated rat with room air inhalation (Sham + room air 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 + room air group, n = 7), tMCAO-operated rat with room air inhalation (tMCAO + room air group, n = 7), and tMCAO-operated rat with intermittent $CO_2$ inhalation (tMCAO + $CO_2$ group, n = 7). To investigate whether $CO_2$ per-conditioning has a beneficial effect on ischemia-reperfusion injury, we conducted additional experiments without randomization on the pMCAO-operated rat with intermittent $CO_2$ inhalation (pMCAO + $CO_2$ group, n = 5) and tMCAO-operated rat with intermittent $CO_2$ inhalation (tMCAO + $CO_2$ group, n = 5).

### *Assessment of neurological functions*

Neurologic evaluation was performed at 24 h after the surgical procedure. We carried out various scoring methods for appropriate assessment – Garcia test, Longa test, rotarod test, and modified Neurological Severity Score (mNSS). Neurologic deficits of the rats were examined by an observer who was blinded on treatment allocation. 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.[28] 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.[29] The rotarod test, animals are placed on the rotarod cylinder, and duration the animals endured on the rotarod is measured.[30,31] The mNSS examined motor, sensory, reflex and balance tests. Neurological function of the mNSS is graded on a scale of 0 to 18.[30,32] The score directly correlates with the severity of the injury.[30]

### *Arterial blood gas analysis*

The ventral artery of the experimental animal's tail served for blood collection. Baseline blood gas levels

were obtained from all rats before surgery. In the $CO_2$ inhalation groups, 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).

### *Measurement of regional cerebral blood flow*

Laser Doppler flowmetry (BLF 21 Laser Doppler flowmeter, Transonic Systems INC., Ithaca, NY, USA) was used to indirectly measure regional CBF in the MCA area. The laser sources were positioned 4 mm lateral and 2 mm posterior to the bregma. To assess changes in CBF, we measured tissue perfusion unit (TPU) at baseline before surgery, after surgery, immediately after $CO_2$ inhalation, and immediately after reperfusion. We observed changes in TPU in both the sham + $CO_2$ and tMCAO + $CO_2$ groups at each intervention time.

### *Infarct size measurement*

At 24 hours after surgery, the experimental animals were euthanized under deep inhalation anesthesia with 5% isoflurane mixed with 70% nitrous oxide and 30% oxygen, and then cardiac perfusion was performed using a peristaltic pump. 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 softwares (Scion Image®, Frederick, MD, USA and Image J, National Institutes of Health, Bethesda, MD, USA). Infarct size was normalized to the contralateral area and expressed as a percentage. Average values from the 4 sections were calculated. Additionally, to compare the rate of peri-infarct swelling in the MCAO groups, we subtracted the size of the contralesional hemisphere from the size of the ipsilesional hemisphere, then divided this value by the size of the contralesional hemisphere and converted it to a percentage for comparison.[33]

### *Immunofluorescence analysis*

Immunofluorescence analysis was conducted using primary antibody of 8-hydroxy-2-deoxyguanosine (8-OHdG) as a marker of oxidative damage to deoxyribonucleic acid (DNA). Additionally, platelet-derived growth factor receptor beta (PDGFRβ) was employed to assess pericytes expression, while matrix metalloproteinase-9 (MMP-9) in combination with PDGFRβ was used to identify MMP-9 positive pericytes. Zonula occludens-1 (ZO-1) was utilized to measure the expression of tight junction proteins. 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 blocked with blocking solution for 1 hour 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) overnight at 4°C. The stained sections were scanned with a confocal microscope (LSM 710, Zeiss Instruments, Oberkochen, Germany). The investigators chose cortical peri-infarct as 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).

### *Western blot analysis*

Proteins from the ipsilateral side of rat brain samples were extracted in homogenization buffer (50 mM Tris, 120 mM NaCl, pH 7.4) containing protease inhibitors separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane. The blocked membranes were incubated overnight at 4°C with the primary antibody. 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 with ImageJ software (v1.4, NIH, Bethesda, MD, USA). The 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%).

### Statistical analysis

All the data in this study were presented as the median and interquartile range (IQR). The Kruskal-Wallis H test was performed to compare the baseline results of whole groups, and the Wilcoxon signed rank test was performed to compare pairwise results of ABGA in groups with $CO_2$ inhalation. We used one-way repeated measures ANOVA followed by post-hoc multiple comparisons with Bonferroni correction to analyze CBF at each time point. The Kruskal-Wallis H test, followed by post-hoc multiple comparisons with Bonferroni correction, was performed on the behavior test scores, cell counts, fluorescence intensities from immunohistochemical staining, and ratios of blot bands. 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) and PRISM 10 software (GraphPad PRISM Software, La Jolla, CA, USA).

## Results

### Physiologic variables before and after $CO_2$ per-conditioning

No rat experienced mortality throughout the experiment. Body weight and rectal temperature were not significantly different among the experimental groups. The pH, $pCO_2$, $pO_2$, and $HCO_3$ levels of baseline ABGA did not show significant difference among the groups (Kruskal-Wallis H test; For pH, $p = 0.8$; for $pCO_2$, $p > 0.9$; for $pO_2$, $p = 0.8$; and for $HCO_3$, $p > 0.9$). Following $CO_2$ inhalation, $pCO_2$ levels were increased (Wilcoxon signed rank test; for sham + $CO_2$ group, $p = 0.062$; and for tMCAO + $CO_2$ group, $p = 0.016$). There were reductions in the pH levels of both the sham + $CO_2$ and tMCAO + $CO_2$ groups (Supplementary Table 1).

### Cerebral blood flow before and after $CO_2$ per-conditioning

In the sham + $CO_2$ group, after surgery, there was a significant decrease in TPU compared to the baseline (mean 60.7 [SD 5.5] at baseline vs. mean 55.9 [SD 4.8] after surgery; $p = 0.016$). Compared to the baseline, $CO_2$ inhalation resulted in a significant increase in TPU (mean 60.7 [SD 5.5] at baseline vs. mean 96.0 [SD 4.2] after $CO_2$ inhalation; $p < 0.001$, Supplementary Figure 2). In the tMCAO + $CO_2$ group, there was a significant decrease in TPU after surgery compared to the pre-surgery time point (mean 58.4 [SD 3.6] at baseline vs. mean 3.6 [SD 0.4] after surgery; $p < 0.001$), and a significant increase immediately after $CO_2$ inhalation (mean 3.6 [SD 0.4] after surgery vs. mean 34.5 [SD 5.5] after $CO_2$ inhalation; $p = 0.001$, Supplementary Figure 2).

### $CO_2$ per-conditioning attenuates the neurological deficit and infarct size after tMCAO

Regarding the behavior tests, the tMCAO + $CO_2$ group showed significantly higher Garcia and rotarod test scores than the tMCAO + room air and pMCAO + room air groups, indicating better neurological function (Figure 2(a) and (b)). While the mNSS and Longa test scores for the tMCAO + $CO_2$ group were lower than those for other MCAO + room air groups, the differences were not statistically significant (Figure 2(c) and (d)).

TTC staining analysis revealed the tMCAO + $CO_2$ group had a significantly smaller infarct size when compared to the tMCAO + room air and pMCAO + room air groups. Infarct sizes were recorded at 14.8% (IQR 11.0–17.8%) for the tMCAO + $CO_2$ group, versus 27.9% (IQR 26.4–28.0%, $p < 0.001$) for the tMCAO + room air group, and 36.1% (IQR 34.9–36.5%, $p < 0.001$) for the pMCAO + room air group (Figure 3 and Supplementary Table 2). The tMCAO + room air group exhibited a significantly smaller infarct size compared to the pMCAO + room air group ($p < 0.001$, Figure 3 and Supplementary Table 2). For peri-infarct swelling, although numerical differences were observed, they did not reach statistical significance among the study groups (tMCAO + $CO_2$ group, 5.3% [SD 2.7]; tMCAO + room air group, 7.1% [SD 3.1]; and pMCAO + room air group, 10.0% [SD 5.0]; Kruskal-Wallis H test, $p = 0.140$, Supplementary Table 3).

### $CO_2$ per-conditioning downregulates peri-lesional oxidative stress after tMCAO

The number of 8-OHdG positive cells was significantly lower in the tMCAO + $CO_2$ group compared to the tMCAO + room air or the pMCAO + room air groups (tMCAO + $CO_2$ vs. tMCAO + room air, $p = 0.008$; and tMCAO + $CO_2$ vs. pMCAO + room air, $p = 0.002$, Figure 4). Furthermore, the pMCAO + room air group exhibited significantly higher count of 8-OHdG positive cells compared to the tMCAO + room air group ($p = 0.002$, Figure 4).

### $CO_2$ per-conditioning alleviates BBB breakdown after tMCAO

In the peri-infarct areas, the tMCAO + $CO_2$ group showed a significant lower count of MMP-9/PDGFRβ double positive cells compared to the other MCAO

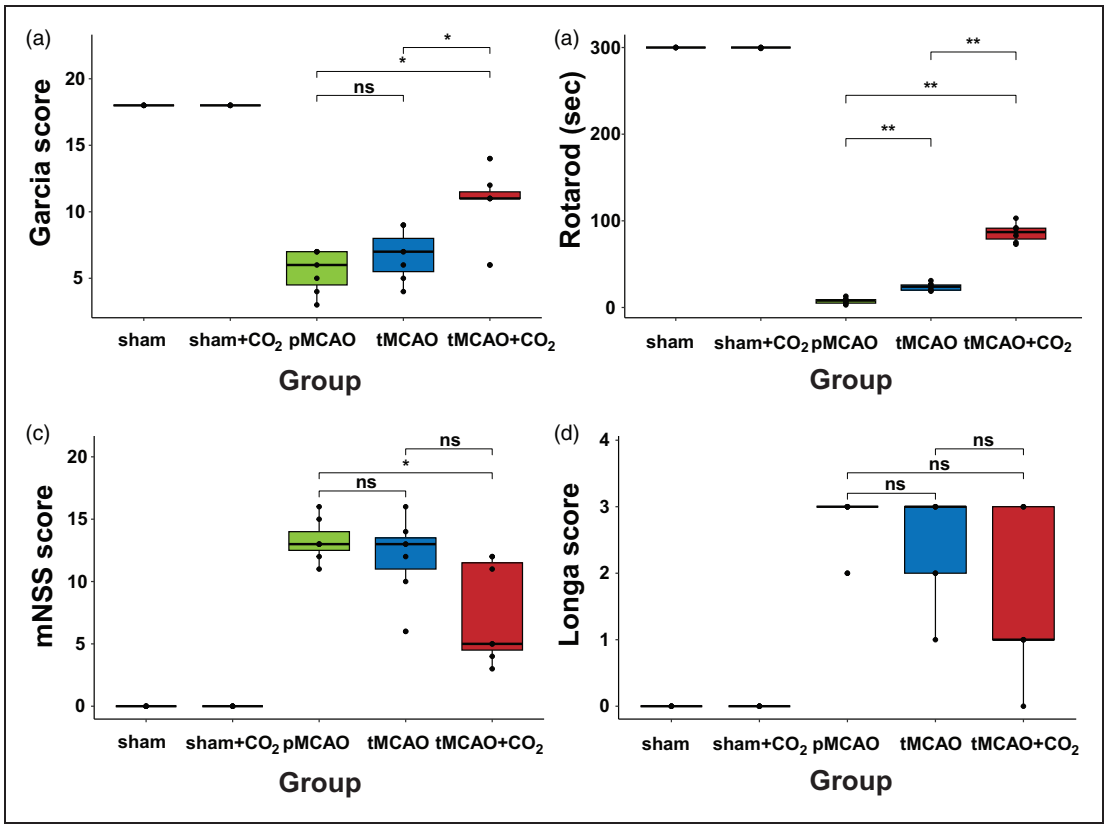


**Figure 2.** Behavior test score of Garcia, Rotarod, mNSS, and Longa tests. (a and b) Garcia and rotarod test score of the tMCAO + $CO_2$ group were significantly higher than those of the other MCAO groups. (c and d) In the mNSS and Longa test, the tMCAO + $CO_2$ groups showed a lower median score compared to the other MCAO groups; however, these differences were not statistically significant (*$p < 0.05$, **$p < 0.01$). $CO_2$: carbon dioxide; pMCAO: permanent middle cerebral artery occlusion; tMCAO: transient middle cerebral artery occlusion.

groups (tMCAO + $CO_2$ vs. tMCAO + room air, $p = 0.033$; and tMCAO + $CO_2$ vs. pMCAO + room air, $p = 0.004$, Figure 5). Compared to the tMCAO + room air group, the pMCAO + room air group revealed a higher expression of MMP-9/PDGFRβ double positive cells ($p = 0.048$, Figure 5).

## *$CO_2$ per-conditioning upregulates tight junction protein expressions after tMCAO*

The quantity of ZO-1 positive cells did not significantly differ between the tMCAO + $CO_2$ and the tMCAO + room air groups ($p = 0.141$, Figure 6(a) and (b)). However, the tMCAO + $CO_2$ group maintained a higher number of ZO-1 positive cells than the pMCAO + room air group ($p = 0.006$, Figure 6(a) and (b)). Western blot analysis showed that the tMCAO + $CO_2$ group had higher protein levels of ZO-1 than the other MCAO + room air groups (tMCAO + $CO_2$ vs. tMCAO + room air, $p = 0.021$; and tMCAO + $CO_2$ vs. pMCAO + room air, $p < 0.001$, Figure 6(c)).

## *$CO_2$ per-conditioning increase pericytes survival after tMCAO*

The tMCAO + $CO_2$ group maintained a higher number of PDGFRβ positive cells compared to the other MCAO + room air groups (tMCAO + $CO_2$ vs. tMCAO + room air, $p = 0.008$; and tMCAO + $CO_2$ vs. pMCAO + room air, $p = 0.006$, Figure 7(a) and (b)). No significant difference was observed in the expression of PDGFRβ-positive cells between the tMCAO + room air group and the pMCAO + room air group ($p = 0.9$, Figure 7(a) and (b)). Western blot analysis indicated that the tMCAO + $CO_2$ group exhibited a trend towards higher PDGFRβ protein levels than the tMCAO + room air groups ($p = 0.072$, Figure 7(c)), and significantly higher levels than the pMCAO + room air groups ($p = 0.020$, Figure 7(c)).

## *$CO_2$ per-conditioning is involved in the process of ischemia-reperfusion injury*

To investigate whether $CO_2$ per-conditioning is involved in ischemia-reperfusion injury, we compared

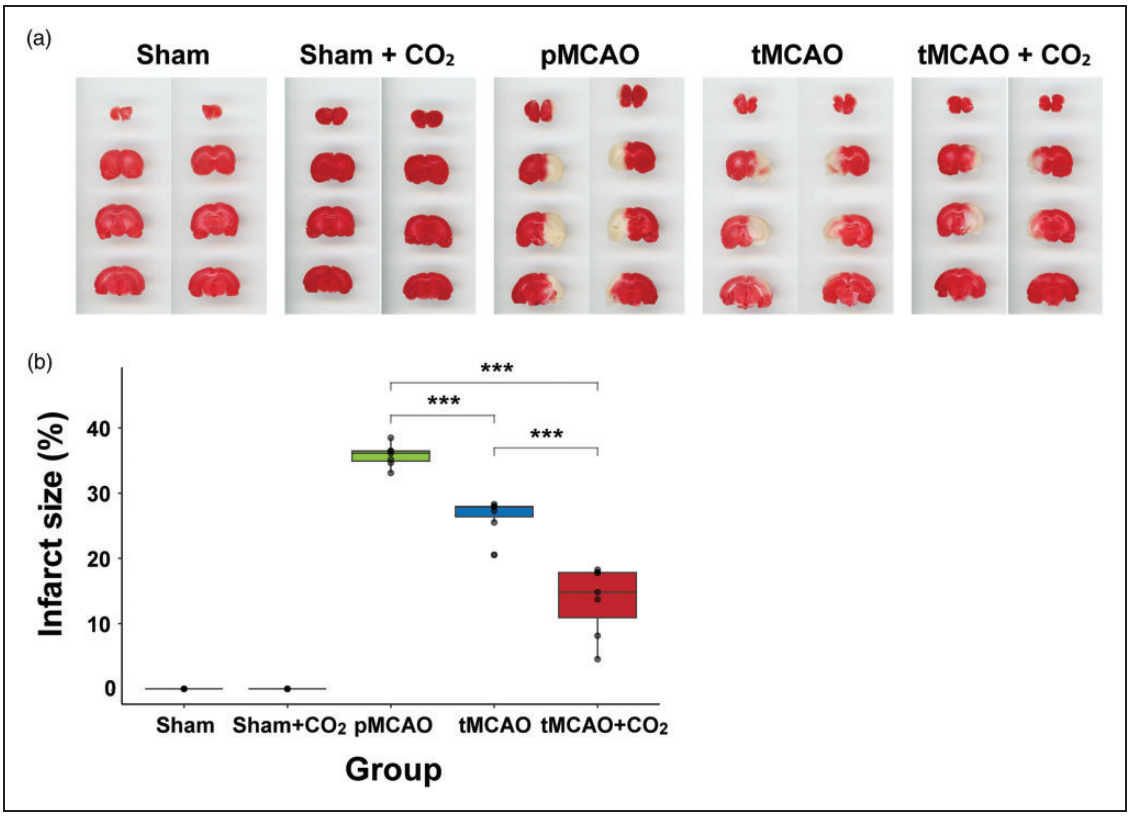


**Figure 3.** Infarct size measurement. (a) The representative TTC-stained images illustrate the infarct sizes across groups, with the tMCAO + $CO_2$ group displaying a smaller infarct area compared to both the tMCAO and pMCAO groups and (b) quantitative analysis of infarct size according to the groups (***$p < 0.001$). Data are reported as median and IQR. $CO_2$: carbon dioxide; pMCAO: permanent middle cerebral artery occlusion; tMCAO: transient middle cerebral artery occlusion; TTC: 2-3-5-triphenyl tetrazolium chloride.

infarct size and behavioral tests between the pMCAO + $CO_2$ group and the tMCAO + $CO_2$ group. In the tMCAO + $CO_2$ group, the infarct size was significantly smaller compared to the pMCAO + $CO_2$ group ($p = 0.002$, Supplementary Figure 3). Although the Longa test did not reach statistical significance ($p = 0.158$), both the Garcia test and mNSS test demonstrated significantly more favorable neurological outcomes in the tMCAO + $CO_2$ group compared to the pMCAO + $CO_2$ group (Garcia test, $p = 0.037$; and mNSS test, $p = 0.010$, Supplementary Figure 3).

## Discussion

In the present study, we demonstrated that neuroprotective effect of mild $CO_2$ per-conditioning in tMCAO rat model. Our data illustrated that $CO_2$ per-conditioning significantly decreased the infarct size and neurologic deficit by reducing the oxidative stress, BBB breakdown, and death of pericytes after tMCAO.

Prior neuroprotection research typically examined pre- or post-conditioning in hyperacute ischemic stroke animal models, but none has explored per-conditioning with mild hypercapnia during the ischemic phase. In this study, we focused on per-conditioning, that can be clinically used before reperfusion by EVT with hyperacute ischemic stroke. EVT is the most efficacious treatment option for hyperacute ischemic stroke due to large vessel occlusion. The ESCAPE-NA1 trial tested the adjunctive nerinetide administration as a neuroprotection agent could improve outcomes in ischemic stroke patients who undergoing EVT for large vessel occlusion.[5] Although the trial failed to show the overall neuroprotective effect of nerinetide, a prespecified subgroup analysis revealed that patients who did not receive alteplase exhibited neuroprotective benefits from nerinetide. The ESCAPE-NA1 trial, using a per-conditioning method similar to ours, showed benefits from administering a neuroprotective agent before EVT. This suggests that per-conditioning with a neuroprotective agent to mitigate reperfusion injury and the no-reflow phenomenon could be a viable strategy in acute ischemic stroke. We hypothesized that both per-conditioning and mild hypercapnia will synergistically work with each other. We employed intermittent $CO_2$ inhalation as a

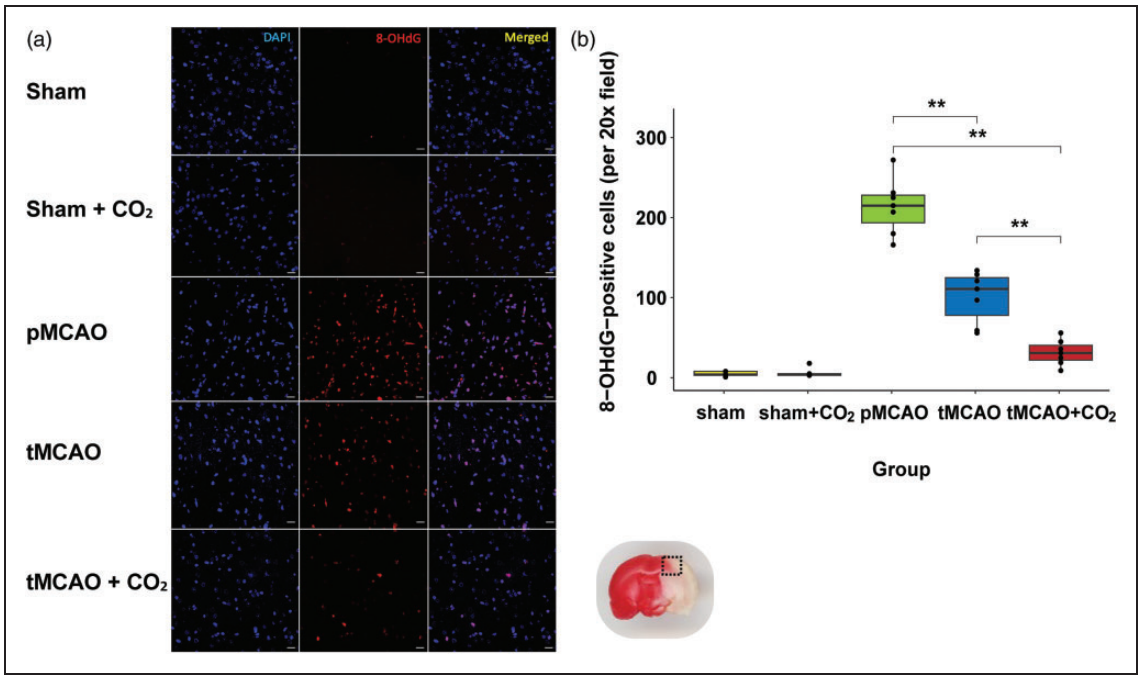


**Figure 4.** $CO_2$ per-conditioning decreased the number of 8-OHdG positive cells. (a) Representative Images of immunofluorescence staining illustrated the 8-OHdG positive cells. scale bar = 20 μm and (b) quantitative analysis of 8-OHdG expression in immunofluorescence staining (**$p < 0.01$). 8-OHdG expressions in all rat models presented similar pattern. 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.

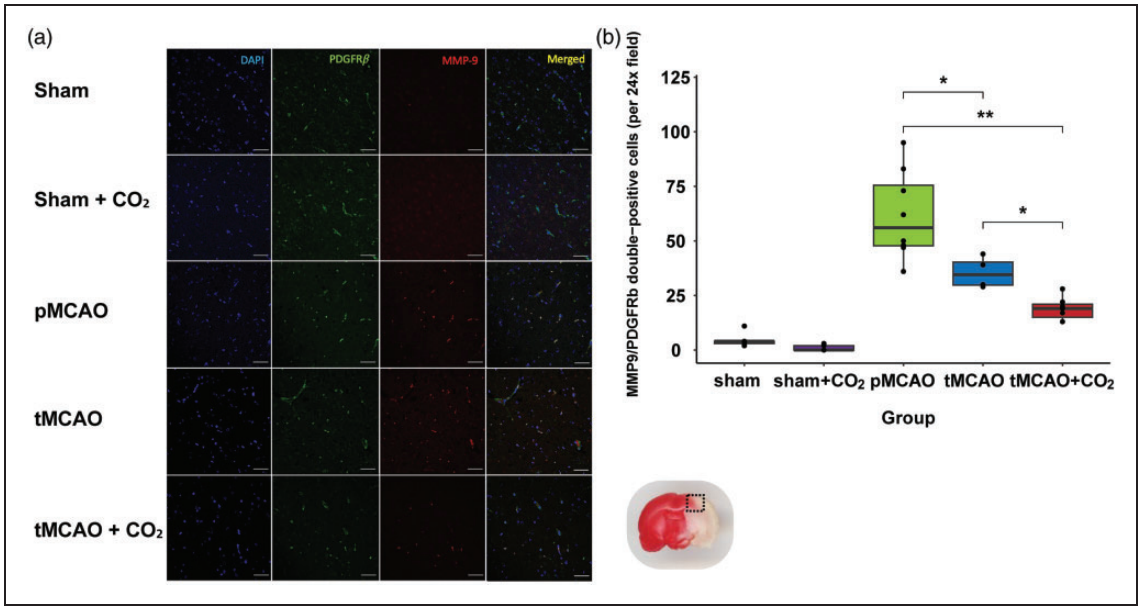


**Figure 5.** $CO_2$ per-conditioning decreased the number of MMP-9/PDGFRβ double positive cells. (a) Representative Images of immunofluorescence staining illustrated the PDGFRβ, MMP-9, and MMP-9/PDGFRβ double positive cells. scale bar = 20 μm and (b) quantitative analysis of MMP-9/PDGFRβ expression in immunofluorescence staining (*$p < 0.05$, **$p < 0.01$). MMP-9 and PDGFRβ expressions in all rat models presented similar pattern. $CO_2$: 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.

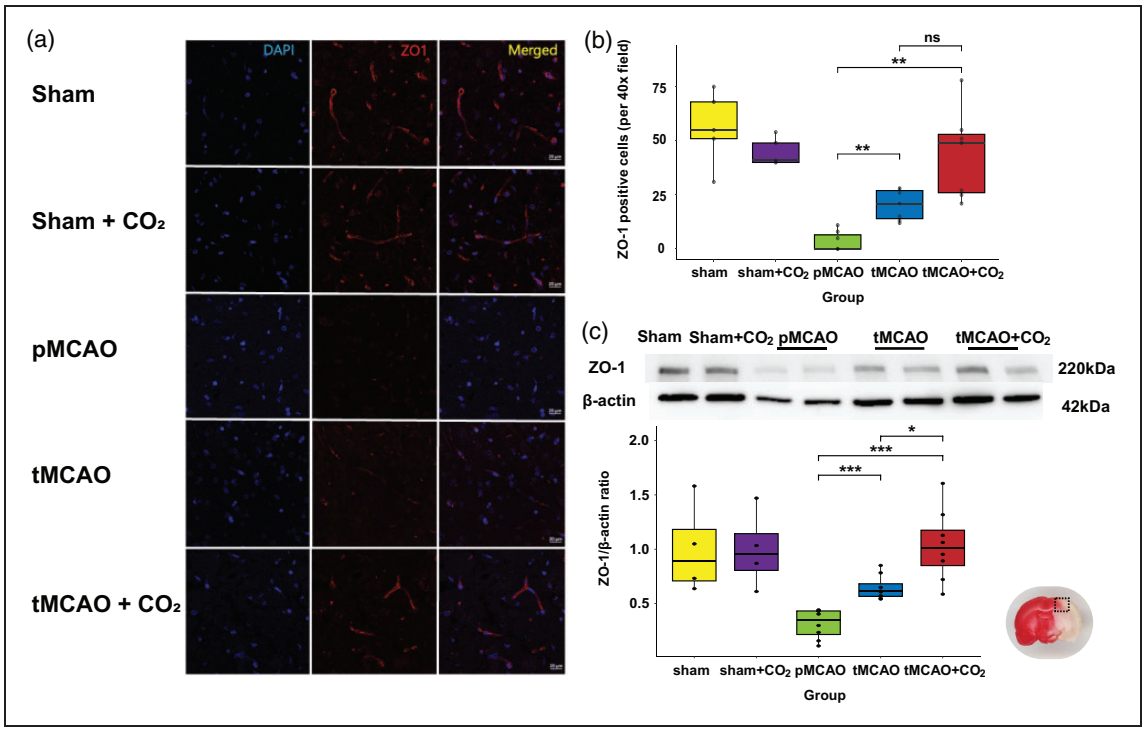


**Figure 6.** $CO_2$ per-conditioning increased the number of ZO-1 positive cells. (a) Representative Images of immunofluorescence staining illustrated the ZO-1 positive cells. scale bar = 20 μm. (b) Quantitative analysis of ZO-1 expression in immunofluorescence staining (**$p < 0.01$) and (c) Western blot analysis of ZO-1 expression (*$p < 0.05$, ***$p < 0.001$). ZO-1 expressions in all rat models presented similar pattern. $CO_2$: 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.

per-conditioning method to evaluate its neuroprotective efficacy.

$CO_2$ is fast acting vasodilatory gas and can cross BBB owing to its fat-soluble characteristics.[34,35] Due to these specific features, $CO_2$ may exert a neuroprotective effect on ischemic state. 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.[36,37] Relaxation of smooth muscles is induced as $PaCO_2$ increases, which mediated through accompanying changes in pH and/or directly by $CO_2$.[11,38,39] When $PaCO_2$ is elevated by 10–20 mmHg above normal resting state, CBF increases by 3–4% for each unit increase in $PaCO_2$.[40] The increase in CBF through vasodilation in a hypo-perfused state may potentially reduce ischemic injury.

In this study, we intermittently inhaled 20% $CO_2$, which is the most efficient $CO_2$ concentration identified in our pilot study (data not shown) and other studies.[11,41] Based on ABGA results of $CO_2$ inhalation groups, the $PaCO_2$ values in our 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 be enough to reverse the salvageable tissue by alleviating the decrease in CBF.[40] In contrast, excessive increase in $CO_2$ concentration resulted in poor outcomes through increased intracranial pressure rather than neuroprotective effects.[42] Therefore, it is critical to maintain adequate mild hypercapnia during ischemia for safety aspect. Recent multinational, multi-center randomized clinical trial in patients with cardiac arrest showed that mild hypercapnia is unlikely to cause clinically relevant elevation in intracranial pressure.[13] The other randomized clinical trial demonstrated beneficial effect of targeted therapeutic mild hypercapnia after cardiac arrest.[14] The latter trial showed that the median difference of $PaCO_2$ between the intervention and control groups was 8 mmHg and this result was like our ABGA results.

We revealed that that $CO_2$ per-conditioning can reduce oxidative stress following cerebral ischemia, as evidenced by the decrease in 8-OHdG levels. Oxidative stress which is caused by excessive production of ROS due to imbalance between pro-oxidants and antioxidants is known as a key deleterious factor in brain ischemia. During the reperfusion phase, while thrombolysis therapy removed the blood clot, blood flow restoration can have detrimental effects due to the

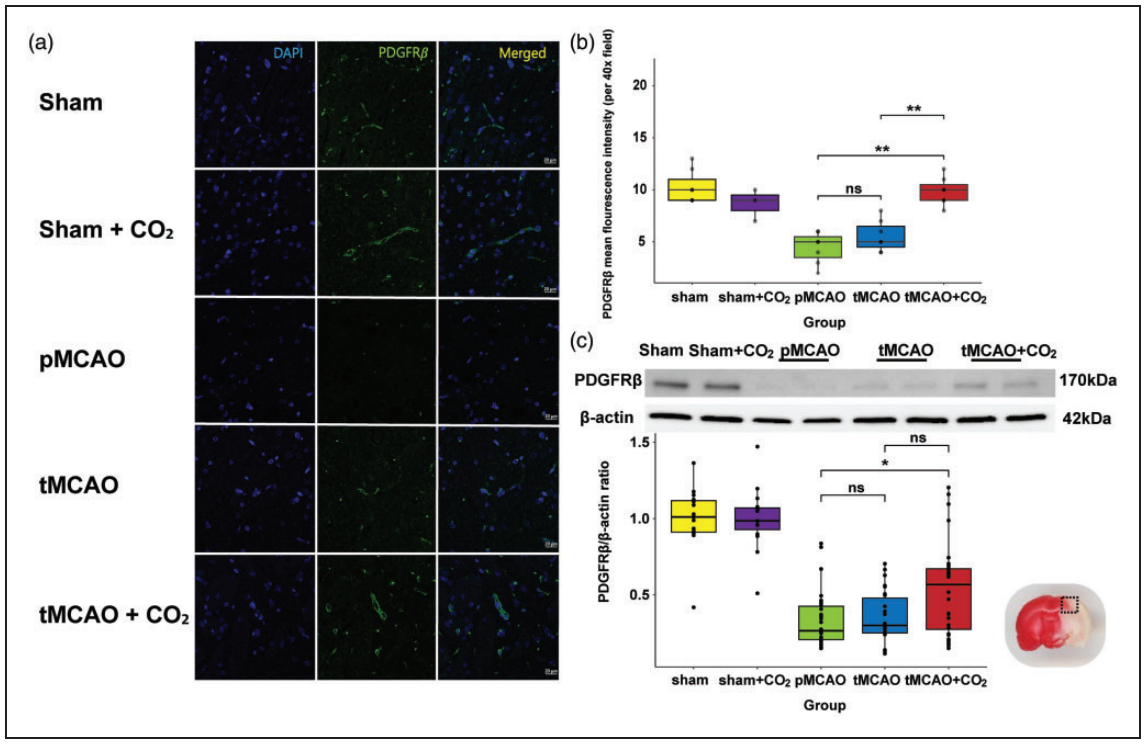


**Figure 7.** $CO_2$ per-conditioning increased the fluorescence intensity of PDGFRβ positive cells. (a) Representative Images of immunofluorescence staining illustrated the PDGFRβ positive cells. scale bar = 20 μm. (b) Quantitative analysis of PDGFRβ expression in immunofluorescence staining (**$p < 0.01$) and (c) Western blot analysis of PDGFRβ expression (*$p < 0.05$). PDGFRβ expressions in all rat models presented similar pattern. $CO_2$: 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.

excessive production of ROS.[43] Reperfusion leads to a surge in superoxide production as $NAD^+$ is converted to NADH within the mitochondrial respiratory chain, and ROS are also generated in the cytoplasm through NADPH-oxidase reactions.[44] One study indicated that $CO_2$ can interact with reactive oxygen species and scavenge the peroxynitrite, which is derived from superoxide, thereby mitigating oxidative stress.[45] This anti-oxidative effect of $CO_2$ may have contributed to the reduction of oxidative stress induced by the ischemia-reperfusion state.

Our results showed that mild hypercapnia attenuated MMP-9 expression and upregulated ZO-1. MMP-9 is well known mediator of BBB breakdown caused by degradation of basement membrane proteins in ischemia-reperfusion injury.[46,47] Furthermore, elevated levels of MMP-9 were correlated with larger infarct size and unsuccessful recanalization.[48,49] These findings suggested that mild hypercapnia can decrease BBB breakdown by attenuating MMP-9 expression and increasing ZO-1 expression. Additionally, our results showing that the tMCAO + $CO_2$ group had numerically smaller peri-infarct swelling compared to the tMCAO or pMCAO groups may also support the finding of reduced BBB breakdown.

Interestingly, we found that mild hypercapnia augmented pericytes survival 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.[23,50,51] In the ischemic state, contraction of pericytes triggers capillary constriction, and then erythrocytes are trapped in microvessels.[25,26] After recanalization of occluded vessels, pericytes remain contracted despite successful reperfusion.[26] It can cause no-reflow phenomenon. One study showed that elevated $PaCO_2$ increase pericytes survival and induce relaxation of pericytes, whereas decreased $PaCO_2$ causes contraction of pericytes.[10] Therefore, we can hypothesize the mechanism of beneficial roles of $CO_2$ may be mediated by pericytes. In addition, because pericytes contraction can also be induced by ROS, the anti-oxidative effect of hypercapnia may relieve pericytes contraction, which triggers capillary constriction.[26]

Mild hypercapnia may have various effects on hypoperfused brain tissue. First, mild hypercapnia may directly increase the pericytes survival and prevent their contraction, which could lessen the no-reflow phenomenon.[10,26] Second, by lowering ROS levels, mild hypercapnia could not only directly reduce the infarct size but also influence pericytes survival and BBB integrity, potentially contributing to neuroprotection.[43,45] Third, mild hypercapnia could decrease BBB breakdown, potentially reducing ischemia-reperfusion injury.[46,48,49] These diverse effects of mild hypercapnia might synergistically contribute to neuroprotective effect. Additionally, since $CO_2$ per-conditioning has been shown to mitigate ischemia-reperfusion injury, $CO_2$ pre- or post-conditioning might also have clinical utility. This warrants consideration for further research.

Due to its minimal reactivity and low risk, $CO_2$ can be used outside of hospital. Early initiation of mild hypercapnia can be considered even before arriving at the hospital. In acute stroke, it is nearly impossible differentiate between cerebral infarction and cerebral hemorrhage without brain imaging. If the safety of mild hypercapnia is confirmed in cerebral hemorrhage, inhalation of $CO_2$ in the ambulance may be promising strategy.

There are several limitations in this study. First, we did not measure concurrent $PaCO_2$ level during intervention and surgery. This may be due to a minimum 10-minute gap ensued between $CO_2$ inhalations. However, follow-up ABGA results after $CO_2$ inhalation showed a lower $PaCO_2$ level than anticipated. In addition, we could not continuously monitor how $PaCO_2$ levels changed physiologically during and after the procedure. Second, stroke exhibits sex-based differences in clinical settings, with protective female sex hormones leading to a lower risk of stroke in women compared to men.[52,53] Differences may also emerge between sexes in experimental stroke models. Therefore, sex difference needs to be considered in further study to determine the effect of intermittent $CO_2$ inhalation in ischemic stroke models. Third, in the additional experiment comparing $pMCAO + CO_2$ group and $tMCAO + CO_2$ group, randomization was not performed. This could have led to a batch effect. Fourth, the $tMCAO + CO_2$ group exhibited baseline hypocapnia before $CO_2$ inhalation. Since baseline hypocapnia can cause vasoconstriction and potentially affect infarct size, particularly during tMCAO induction, it may be needed to exclude animals with baseline hypocapnia. However, excluding animal after randomization may potentially bring bias. Additionally, our findings suggest that $CO_2$ inhalation provided neuroprotective benefits that outweighed the possible negative effects of baseline hypocapnia. Lastly, the observation period was relatively limited (24 hours). In clinical practice, the modified Rankin Scale assesses disability or dependence levels after a stroke, typically weeks or months post-event. Given a rat's lifespan approximates two weeks to a human year, evaluations need to be extended to a week or more. Future research needs to be conducted to assess the structural and functional changes in recovery phase after ischemic stroke over weeks to months.

## Conclusion

Mild hypercapnia before reperfusion decreases infarct size and neurologic deficit. Per-conditioning with intermittent inhalation of $CO_2$ preserves BBB integrity and neurovascular unit, reduces oxidative stress, and pericytes survival in a hyperacute ischemic stroke rat model. Therapeutic hypercapnia before reperfusion is promising and requires further assessment for clinical application.

### Data availability

Request for data collected for the study can be made to the corresponding author and will be considered on reasonable request.

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### Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

### Authors' contributions

J.W.J., C.E.Y., and H.S.N designed the study and wrote the manuscript. J.W.J. C.E.Y., and H.S.N analyzed the data. J.W.J., C.E.Y., I.K., K.O.L., J.K., Y.D.K., J.H.H., and H.S.N participated in data collection and contributed to writing the paper.

### Supplementary material

Supplemental material for this article is available online.

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