Background and Purpose—Prolonged ischemia causes blood–brain barrier (BBB) damage and increases the incidence of neurovasculature complications secondary to reperfusion. Therefore, targeting ischemic BBB damage pathogenesis is critical to reducing neurovasculature complications and expanding the therapeutic time window of tissue-type plasminogen activator (tPA) thrombolysis. This study investigates whether increasing cerebral tissue PO2 through normobaric hyperoxia (NBO) treatment will slow the progression of BBB damage and, thus, improve the outcome of delayed tPA treatment after cerebral ischemia.

Methods—Rats were exposed to NBO (100% O2) or normoxia (21% O2) during 3-, 5-, or 7-hour middle cerebral artery occlusion. Fifteen minutes before reperfusion, tPA was continuously infused to rats for 30 minutes. Neurological score, mortality rate, and BBB permeability were determined. Matrix metalloproteinase-9 was measured by gelatin zymography and tight junction proteins (occludin and claudin-5) by Western blot in the isolated cerebral microvessels.

Results—NBO slowed the progression of ischemic BBB damage pathogenesis, evidenced by reduced Evan blue leakage, smaller edema, and hemorrhagic volume in NBO-treated rats. NBO treatment reduced matrix metalloproteinase-9 induction and the loss of tight junction proteins in ischemic cerebral microvessels. NBO-afforded BBB protection was maintained during tPA reperfusion, resulting in improved neurological functions, significant reductions in brain edema, hemorrhagic volume, and mortality rate, even when tPA was given after prolonged ischemia (7 hours).

Conclusions—Early NBO treatment slows ischemic BBB damage pathogenesis and significantly improves the outcome of delayed tPA treatment, providing new evidence supporting NBO as an effective adjunctive therapy to extend the time window of tPA thrombolysis for ischemic stroke. (Stroke. 2015;46:00-00. DOI: 10.1161/STROKEAHA.114.008599.)

Key Words: blood-brain barrier • ischemia • matrix metalloproteinase 9 • tissue plasminogen activator

Thrombolysis with tissue-type plasminogen activator (tPA) is the only Food and Drug Administration–approved therapy for acute ischemic stroke, but it has a narrow therapeutic time window of 3 to 4.5 hours after stroke onset.1 Delayed tPA treatment leads to severe complications, such as intracerebral hemorrhage (ICH) and edema, because of reperfusion into weakened or necrotic brain microvasculature.2,3 Blood–brain barrier (BBB) damage after ischemic stroke is a dynamic process with an initial damage during ischemia and a secondary injury during reperfusion.2,3 Prolonged ischemia leads to severe BBB damage that dramatically increases the risk of complications after tPA thrombolysis. Therefore, preserving BBB integrity during ischemia is of critical importance in ensuring that the microvasculature will safely withstand the return of blood flow.

Oxygen therapy aimed at increasing tissue partial pressure of oxygen (Po2) has long been considered a logic treatment for ischemic stroke.7 Several animal studies have shown that short duration of normobaric hyperoxia (NBO) treatment is highly neuroprotective if started early after ischemia onset.8–12 A pilot clinical study also demonstrated that NBO therapy was associated with a transient improvement of clinical deficits and magnetic resonance imaging abnormalities in patients with acute ischemic stroke.13 These studies have demonstrated that early NBO treatment can slow the progression of ischemic brain tissue to necrosis, thus improving the therapeutic time window for reperfusion therapies. We have shown that besides neuroprotection, NBO can also protect the BBB against ischemic damage through inhibiting reactive oxygen species production14,15 and matrix metalloproteinase-9 (MMP-9)–mediated...
degradation of tight junction proteins (TJPs)\textsuperscript{16–18}. However, the time window for vasoprotection of NBO remains unknown.

In this study, we investigated how, and to what extent, early NBO treatment could slow the progression of ischemic BBB damage using a rat model of focal cerebral ischemia. In addition, we investigated whether the vasoprotection afforded by NBO during cerebral ischemia was effective in reducing the neurovascular complications associated with delayed tPA treatment.

Materials and Methods

Animal Model of Focal Cerebral Ischemia

The Institutional Animal Care and Use Committee of Capital Medical University approved all animal experiments. Male Sprague-Dawley rats (n=144) weighing 290 to 320 g were anesthetized with 2% isoflurane and subjected to middle cerebral artery occlusion (MCAO) followed by reperfusion using the suture occlusion method, as we previously described\textsuperscript{18}. Successful MCAO was confirmed by 2,3,5-triphenyltetrazolium chloride staining\textsuperscript{18}.

Although suture model is not directly relevant to thromboembolic ischemia in patients, this stable model would allow us to compare the different effects of NBO/normoxia on tPA's complications under identical and controlled ischemia and reperfusion conditions. In contrast, the more clinically relevant model of injecting a preformed clot into MCA is not most suitable for this investigative study because we cannot predict how long and where the clot will occlude MCA.

Experimental Design

To investigate the NBO's ability to slow the progression of ischemic BBB damage and its effect on the neurovascular complications of delayed tPA treatment, we chose 3 ischemia durations: 3 hours (within the established 3–4.5 hours thrombolytic time window), 5 hours, and 7 hours (outside the window). We chose 2 reperfusion time points, 2 hours after reperfusion onset and 24 hours after MCAO onset. tPA was intravenously infused for 30 minutes at 15 minutes before reperfusion, mimicking tPA thrombolysis in clinical settings.

Rats with successful MCAO were randomly assigned to 4 groups: normoxia+MCAO (n=30), NBO+MCAO (n=30), normoxia+tPA+MCAO (n=27), and NBO+tPA+MCAO (n=27). Each group was further divided into 3 subgroups with 3-, 5-, or 7-hour MCAO followed by 2-hour reperfusion. Another 30 rats were randomly assigned into 4 groups to assess mortality rate at 24 hours after MCAO normoxia-I. Data are expressed as mean±SEM (n=5).

NBO Treatment

All animals exhibited rapid recovery from anesthesia within 5 minutes post MCAO surgery. When rats could freely move at 10 minutes post MCAO onset, we put them into an anesthesia box that was ventilated (5 L/min) with air (21% O\textsubscript{2}, normoxia) or 100% O\textsubscript{2} (NBO) until 30 minutes before the end of MCAO. 100% O\textsubscript{2} was chosen as the NBO treatment because our previous study demonstrated that rats breathing this O\textsubscript{2} concentration were able to maintain the ischemic penumbral P\textsubscript{O\textsubscript{2}} close to the preischemic level\textsuperscript{18}. For convenience and precise control, the rats were taken out of NBO exposure box for tPA infusion 30 minutes before reperfusion, thus terminating NBO treatment.

Measurement of Neurological Deficits

At the end of 2-hour reperfusion, neurological deficits were assessed double blinded with 2 methods: Zea-Longa score\textsuperscript{11} and Ludmila Belayev\textsuperscript{12}.

Tissue Collection and Measurement of Infarction and Edema

At the end of 2-hour reperfusion, the rats were transcardially perfused with 250 mL of cold PBS. Brains were sectioned into six 2-mm coronal slices from a 12-mm thick region 3 mm away from the tip of the frontal lobe. The third slice was stained using 1% 2,3,5-triphenyltetrazolium chloride to measure the infarct size. The 2-mm thick brain slices were digitally photographed to assess brain edema.

An indirect method for calculating infarct volume was used to minimize error introduced by edema\textsuperscript{23}. The noninfarcted region in the
ipsilateral hemisphere was subtracted from that in the contralateral hemisphere. The infarct size was presented as a percentage of the contralateral hemisphere.

**Evaluation of EB Leakage**
EB (2% w/vol in PBS, 3 mL/kg; Sigma) was administered intravenously in the tail vein at the onset of reperfusion. At the end of 2-hour reperfusion, the rats were transcardially perfused with PBS. The brain was then removed, sectioned, and photographed to visualize EB extravasation. BBB disruption was quantitatively assessed by measuring EB contents in ischemic hemispheric tissue as previously reported.16

**Spectrophotometric Assay of Cerebral Hemorrhage**
Cerebral hemorrhage was quantified using QuantiChrom Hemoglobin Assay Kit (BioAssay Systems, Hayward) as previously described.24

**Measurement of Brain Edema**
Brain edema was assessed by measuring the hemispheric areas of each 2-mm thick brain slice on the digital photographs obtained using Image J software (National Institutes of Health) as described previously.14 Edema was expressed as a ratio of ischemic hemispheric area versus nonischemic hemispheric area.

**Isolation of Cerebral Microvessels**
Isolation of cerebral microvessels was performed as described in our previous study.14

**Measurement of MMP-9, Occludin, and Claudin-5 in Isolated Microvessels**
MMP-9 levels were measured by gelatin zymography, and occludin and claudin-5 protein levels were measured by Western blot in the isolated microvessels, as described in our previous studies.14,17

**Statistical Analysis**
Data were presented as mean±SEM. Statistical analysis was carried out using $X^2$ test and 1-way ANOVA. A value of $P<0.05$ was considered statistically significant.

**Results**

**NBO Slows the Evolution of Ischemic BBB Damage**
To determine whether NBO could slow down the evolution of ischemic BBB damage, we compared the severity of BBB damage with or without NBO treatment after 3, 5, or 7 hours of ischemia followed by 2-hour reperfusion. Figure 1B shows the EB extravasation after 3, 5, or 7 hours of cerebral ischemia. As expected, EB contents in nonischemic hemispheric tissue were low under all tested stroke conditions (Figure 1B). Cerebral ischemia drastically increased EB content in the ischemic hemispheric tissue of the normoxic group, and greater EB leakage was seen for longer ischemic duration. NBO treatment decreased EB extravasation for all 3 ischemic durations with the largest reduction occurring for 7-hour ischemia.

ICH was seen in the ischemic hemisphere of normoxic rats after ischemia, with longer ischemia durations inducing greater bleeding volumes (Figure 2A). Similar to EB extravasation, NBO treatment reduced the severity of ICH for all 3 ischemic durations. A small amount of hemoglobin was detected in the nonischemic hemisphere of all rats, likely because of residual blood left in the cerebral vasculature after transcardial perfusion.

We also assessed brain edema and observed that longer ischemia durations induced more severe brain edema in normoxic rats (Figure 2B). When the rats were treated by NBO during cerebral ischemia, the hemispheric enlarging process appeared to be frozen at the level of 3-hour ischemia because the edematous volume remained unchanged even if the ischemic duration was prolonged to 7 hours.

These results clearly indicate that NBO treatment can slow the evolution of ischemic BBB damage, potentially expanding the therapeutic time window.
NBO Slows the Process of MMP-9 Induction and the Loss of TJs in Ischemic Microvessels

We next studied the mechanisms by which NBO treatment slowed the evolution of ischemic BBB damage. MMP-9 induction and increased TJP degradation are 2 well-studied events critically contributing to BBB damage in ischemic stroke.25 MMP-9 band was faint in nonischemic microvascular extracts of all groups (Figure 3A). MMP-9 induction increased with increasing ischemic duration in the ischemic microvessels. Concurrently, both occludin and claudin-5 protein levels in the microvessels decreased with increasing ischemic duration (Figure 3B). Importantly, NBO treatment attenuated MMP-9 induction and restored the loss of occludin and claudin-5 protein in ischemic microvessels. These findings suggest that NBO treatment can slow the process of MMP-9 induction and the loss of TJs in ischemic microvessels.

NBO Slows MMP-9 Induction and TJP Loss in Ischemic Microvessels After Delayed tPA Administration

The results above clearly showed that NBO treatment could slow the evolution of BBB damage and inhibit 2 key BBB-damaging events, ie, MMP-9 induction and TJP degradation, for a prolonged time window. We wondered whether NBO-afforded BBB protection would remain after delayed tPA treatment and thus expanding tPA’s therapeutic time window. To test this, we assessed MMP-9 induction, TJP protein loss, EB extravasation, hemorrhagic volume, and brain edema under the same experimental regimen as described above except that tPA was administered at the end of each indicated ischemic duration.

Figure 3. Normobaric hyperoxia (NBO) slows the process of matrix metalloproteinase (MMP)-9 induction and the loss of tight junction proteins in ischemic microvessels after 3, 5, or 7 hours of middle cerebral artery occlusion with 2-hour reperfusion. A, Representative gelatin zymography showing MMP-9 activity in isolated microvessels from nonischemic (Non-I) and ischemic (I) hemisphere, and the quantitative analysis results. STD: the standard of MMP-9. *P<0.05 vs the normoxia treated with the same ischemic duration. B, Representative western blots of occludin and claudin-5 and the quantitative analysis results. *P<0.05 vs the normoxia treated with the same ischemic duration. Data are expressed as mean±SEM (n=5).

Administration of tPA did not affect MMP-9 levels in the nonischemic microvessels of the normoxic rats (Figure 4A). However, the induction of MMP-9 was elevated and saturated in normoxic rats after tPA administration because all 3 ischemic durations induced the same level of MMP-9 induction. Importantly, NBO treatment abolished MMP-9 induction in the ischemic microvessels of tPA-treated rats for all 3 ischemic durations.

As expected, ischemia caused an ischemia duration–dependent loss of occludin and claudin-5 proteins in ischemic microvessels after tPA administration (Figure 4B). Interestingly, NBO treatment did not reduce occludin and claudin-5 loss induced by 3-hour MCAO plus tPA administration; however, it prevented the further loss of these proteins in rats with 5- and 7-hour ischemic durations.

NBO Slows Down the Deterioration of BBB in Delayed tPA Treatment

Cerebral ischemia with tPA administration led to an ischemia duration dependently increased in EB extravasation in ischemic tissue (Figure 5A). NBO treatment reduced EB...
extravasation in tPA-treated rats with 3-, 5-, or 7-hour MCAO. Similarly, hemoglobin contents in the ischemic tissue of normoxia+tPA-treated rats increased in an ischemia duration–dependent manner, whereas NBO treatment reduced cerebral hemorrhage in tPA-treated rats (Figure 5B). Brain edema was progressively worsened with prolongation of MCAO durations, and NBO treatment slowed this deteriorating process (Figure 5C). These results indicated that NBO-afforded BBB protection during cerebral ischemia was sustained after tPA administration.

**Discussion**

The clinical use of tPA has been profoundly constrained because of the narrow therapeutic time window and the potentially devastating hemorrhagic complications. Previous studies showed that NBO given during cerebral ischemia is neuroprotective and vasoprotective in a rat model of transient cerebral ischemia.\(^{10,14–16,18,26}\) Here, we demonstrated that NBO treatment effectively slowed the progression of ischemia-induced BBB disruption, thus expanding the window of opportunity for other drug treatment. Using tPA as an example, we showed that comparing with tPA alone, combined NBO/tPA treatment led to significant improvement in neurological outcomes and reductions in brain edema, ICH severity, and mortality rate after delayed tPA treatment, despite prolonged ischemia duration (≤7 hours). Our results suggest that NBO could be used as an adjunct therapy to expand the therapeutic time window of tPA thrombolysis for ischemic stroke.

To maximize tPA’s benefit in treating patients with acute stroke, 2 conditions have to be met at the time of tPA administration: a salvageable penumbra and low risk of severe neurovascular complications. Any treatment that can slow the evolution of ischemic penumbra to irreversible injury...
or reduce tPA's neurovascular complications would serve as an effective adjuvant therapy to improve the efficacy of tPA thrombolysis. We and others have shown that NBO given during cerebral ischemia can preserve the ischemic penumbra.\textsuperscript{10,26–28} We have also demonstrated that NBO could protect the BBB against ischemic damage.\textsuperscript{14–18} In this context, NBO has been suggested as an ideal adjunct therapy to improve tPA's efficacy and safety.\textsuperscript{7,8,29} Here, our findings show that NBO treatment can effectively reduce EB extravasation, cerebral hemorrhage, and brain edema despite prolonged ischemia of ≤7 hours, indicating that NBO-afforded BBB protection is sustained after tPA administration. These results indicate that NBO may have the potential to serve as an adjuvant therapy to safely widen the narrow time window of tPA thrombolysis for ischemic stroke.

MMP-9 is induced in ischemic brain tissue and critically contributes to BBB disruption, brain edema formation, and ICH via proteolytic degradation of BBB structural components, including TJP5.\textsuperscript{30–32} Here, our data show that MMP-9 is induced in ischemic cerebral microvessels in an ischemic duration–dependent manner, and this change is accompanied by the loss of the TJP occludin and claudin-5. NBO treatment ameliorated MMP-9 induction and TJP loss. Interestingly, in NBO-treated rats, the changes of MMP-9 and TJP5 appeared to be frozen to a degree observed for 3-hour MCAO because no significant further changes were observed for longer ischemic durations, ie, 5- and 7-hour MCAO. Interestingly, although NBO significantly decreased the infarct volume when compared with the normoxia group, it did not freeze it at 3-hour MCAO level, suggesting that (1) vasoprotective and neuroprotective effects of NBO are likely because of 2 separate processes and (2) NBO treatment affords greater vasoprotection than neuroprotection. Future work is warranted to understand this unexpected finding.

In conclusion, our findings demonstrate that NBO treatment can slow the progression of BBB damage even during prolonged periods of cerebral ischemia, and inhibiting MMP-9 induction and TJP degradation may account for this protection. More importantly, NBO-afforded BBB protection sustains after

\textbf{Figure 5.} Normobaric hyperoxia (NBO) slows the Evan blue (EB) extravasation and hemoglobin extravasation and reduces brain edema in delayed tissue-type plasminogen activator (tPA) treatment. A, Representative brain slices showing EB leakage and the quantitative analysis result in rats with 3, 5, or 7-hour ischemia followed by 2-hour reperfusion. *\(P<0.05\) vs normoxia/tPA+3-hour middle cerebral artery occlusion (MCAO); #\(P<0.05\) vs normoxia–tPA-I+7-hour MCAO. Data are expressed as mean±SEM (n=5). B, Representative brain slices showing hemoglobin extravasation and edema in tPA-treated rats, and the quantitative analysis results. *\(P<0.05\) vs normoxia/tPA+3-hour MCAO; #\(P<0.05\) vs normoxia–tPA treated with the same ischemic duration (n=4). C, The ratio of edema. #\(P<0.05\) vs the tPA treated with the same ischemia duration (n=7).
tPA administration, which results in significant reductions in the neurovascular complications associated with delayed tPA treatment and improved neurological function. However, rationally designed clinical studies with well-defined patient population are needed to validate whether NBO is a viable, safe, and efficacious adjuvant therapy for ischemic stroke.

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Disclosures
None.

References


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