Normobaric Hyperoxia Reduces Blood Occludin Fragments in Rats and Patients With Acute Ischemic Stroke

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Background and Purpose—Damage of the blood–brain barrier (BBB) increases the incidence of neurovascular complications, especially for cerebral hemorrhage after tPA (tissue-type plasminogen activator) therapy. Currently, there is no effective method to evaluate the extent of BBB damage to guide tPA use. Herein, we investigated whether blood levels of tight junction proteins could serve as biomarker of BBB damages in acute ischemic stroke (AIS) in both rats and patients. We examined whether this biomarker could reflect the extent of BBB permeability during cerebral ischemia/reperfusion and the effects of normobaric hyperoxia (NBO) on BBB damage.

Methods—Rats were exposed to NBO (100% O₂) or normoxia (21% O₂) during middle cerebral artery occlusion. BBB permeability was determined. Occludin and claudin-5 in blood and cerebrovascular endothelial cells were measured. Patients with AIS were assigned to oxygen therapy or room air for 4 hours, and blood occludin and claudin-5 were measured at different time points after stroke.

Results—Cerebral ischemia/reperfusion resulted in the degradation of occludin and claudin-5 in microvessels, leading to increased BBB permeability in rats. In blood samples, occludin increased with 4-hour ischemia and remained elevated during reperfusion, correlating well with its loss from ischemic cerebral microvessels. NBO treatment both prevented occludin degradation in microvessels and reduced occludin levels in blood, leading to improved neurological functions in rats. In patients with AIS receiving intravenous tPA thrombolysis, the blood occludin was already elevated when patients arrived at hospital (within 4.5 hours since symptoms appeared) and remained at a high level for 72 hours. NBO significantly lowered the level of blood occludin and improved neurological functions in patients with AIS.

Conclusions—Blood occludin may be a clinically viable biomarker for evaluating BBB damage during ischemia/reperfusion. NBO therapy has the potential to reduce blood occludin, protect BBB, and improve outcome in AIS patients with intravenous tPA thrombolysis.

Clinical Trial Registration—URL: http://www.clinicaltrials.gov. Unique identifier: NCT02974283.

Visual Overview—An online visual overview is available for this article. (Stroke. 2017;48:00-00. DOI: 10.1161/STROKEAHA.117.017713.)

Key Words: blood-brain barrier ■ humans ■ occludin ■ oxygen ■ stroke

Early thrombolytic therapy is critical for the treatment of patients with acute ischemic stroke (AIS). However, thrombolysis significantly increases the risk of hemorrhagic transformation in patients with AIS.1 Studies have shown that ischemia/reperfusion-induced blood–brain barrier (BBB) damage is the main reason for hemorrhagic transformation after vascular recanalization.2,3 Therefore, it is vital to find a reliable biomarker to reflect the progress of BBB permeability changes during cerebral ischemia, helping to evaluate the risk of hemorrhagic transformation and decide whether to apply thrombolysis.

To date, several proteins in serum (matrix metalloproteinase-9, cellular fibronectin, plasminogen activator inhibitor-1, thrombin-activatable fibrinolysis inhibitor, glial fibrillary acidic protein, neuronspecific enolase, and S100 calcium binding protein B) have been reported to be linked to the brain injury after cerebral ischemia.4,5 However, there has been no...
reliable biomarker to accurately reflect the change of BBB permeability during cerebral ischemia.

Tight junction proteins (TJPs), including claudin-5 and occludin, that seal the gap between endothelial cells are important structural components to maintain BBB integrity. The loss of TJPs causes BBB damage and hemorrhagic transformation after cerebral ischemia. Our recent study demonstrated that the fragments of cleaved occludin may release into circulation, and the levels of blood occludin correlate well with the extent of BBB damage in a permanent cerebral ischemic model of rats, suggesting that occludin may serve as a potential biomarker for evaluating BBB damage during the ischemic phase. In this study, we investigated the relationship between TJPs (claudin-5 and occludin) and BBB damage using an ischemia/reperfusion rat model, which better mimics the ischemia reperfusion in clinic. Then we examined the changes of claudin-5 and occludin in serum of patients with ischemic stroke designated to receive intravenous tPA (tissue-type plasminogen activator) thrombolysis.

An ideal biomarker should also respond to the protective effects of therapy. Studies in animals and patients have shown that short duration of normobaric hyperoxia (NBO) treatment is highly neuroprotective if started early after ischemia onset. Animal experiments by our laboratory and others have suggested that NBO can protect BBB by reducing the degradation of TJPs in ischemic microvessels. In this study, we also investigated whether NBO treatment could reduce the level of claudin-5 or occludin in serum both in ischemia/reperfusion rats and in AIS patients with intravenous tPA thrombolysis.

Materials and Methods

Animal Preparation

The Laboratory Animal Care and Use Committee of the Capital Medical University approved all animal experiments. Male Sprague-Dawley rats 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 model, as described previously. Rats in sham group received the same surgical procedures as those in model group, but the suture was not advanced 2 mm away from the tip of the frontal lobe. Ischemic hemispheric tissue was then collected from each brain slice for cerebral microvessel isolation.

NBO Treatment of Rats

Animals recovered rapidly from anesthesia within minutes post-MCAO surgery and were placed into an anesthesia box that was ventilated (5 L/min) with air (21% O2, normoxia) or 100% O2 (NBO) until 10 minutes before the end of MCAO.

Animal Study Design

A total of 128 rats were used in this study, of which 11 were excluded from our analysis because of incomplete occlusion or subarachnoid hemorrhage. To investigate the change of blood claudin-5 and occludin during cerebral ischemia and reperfusion with or without NBO treatment, 36 rats with successful MCAO were randomly assigned into claudin-5 and occludin group with each having 3 subgroups (n=6): sham group, MCAO+normoxia, and MCAO+NBO. In each group, blood samples were collected at 5 time points: 0 hours (basal level), 2-hour MCAO, 4-hour MCAO, 4-hour MCAO+5-minute reperfusion, and 4-hour MCAO+2-hour reperfusion. For measurement of occludin and claudin-5 on microvessels, another 28 rats were randomly assigned into the following groups (n=4): sham group, MCAO+normoxia, and MCAO+NBO group with each having 3 subgroups: 2-hour MCAO, 4-hour MCAO, and 4-hour MCAO+2-hour reperfusion. Brain tissues were collected for microvessel isolation and protein assays. To evaluate the degree of BBB damage and neurological deficits, another 20 rats were randomly assigned into 4 groups (n=5): 2-hour MCAO+2-hour reperfusion (+/- NBO) and 4-hour MCAO+2-hour reperfusion (+/- NBO). To assess 24-hour mortality rate, another 33 rats were randomly assigned into 4 groups: 2-hour MCAO+normoxia (n=8); 2-hour MCAO+NBO (n=8); 4-hour MCAO+normoxia (n=8); and 4-hour MCAO+NBO (n=9).

Evaluation of BBB Permeability by Evan Blue Leakage

Evan blue dye (EB, 2% 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, EB in vessels were removed by transcardially perfusing with PBS. The brain was removed and sectioned to visualize EB extravasation. BBB permeability was assessed by detecting EB contents in ischemic hemispheric tissue, as reported previously.

Brain Tissue Collection

The rats were transcardially perfused with 250 mL cold PBS. Brains were quickly removed and sectioned to 2-mm-thick coronary slices, 2 mm away from the tip of the frontal lobe. Ischemic hemispheric tissue was then collected from each brain slice for cerebral microvessel isolation.

Cerebral Microvessel Isolation

Cerebral microvessels were isolated from ischemic hemisphere, as described in our previous study. Briefly, the hemispheric brain tissue was homogenized in ice-cold PBS. The homogenates were filtered through a 41-μm nylon mesh. Microvessels that retained on the mesh were purified with Dextran T-500 and stored at −80°C for Western blot.

Western Blot Analysis of Occludin and Claudin-5 Protein in Cerebral Microvessels

Isolated cerebral microvessels were incubated in 100 μl RIPA lysis buffer (CST) on ice and centrifuged at 16000 g for 15 minutes at 4°C. The supernatants were collected, and the protein concentrations were determined. Occludin and claudin-5 proteins levels were measured by Western blot, as described in our previous studies. Anti-occludin and anti-claudin-5 were from Invitrogen, and anti-β-actin was from Santa Cruz (CA).

Blood Sample Collection and ELISA Assay

One milliliter of blood was taken from the left femoral vein at each time point: before MCAO, 2-hour MCAO, 4-hour MCAO, 4-hour MCAO+5-minute reperfusion, and 4-hour MCAO+2-hour reperfusion. Serum was separated by centrifugation at 3000 rpm for 10 minutes at 4°C. About 200 μl serum could be harvested to measure the levels of occludin and claudin-5 using commercially available ELISA kits for rat samples (occludin: USCN, China; claudin-5: USCN, China).

Measurement of Neurological Deficits and Mortality Rate

Neurological deficits of rats were determined in a double-blinded manner with Zea-Longa scores at 2-hour MCAO, 4-hour MCAO, and 4-hour MCAO+2-hour reperfusion in each group. Mortality was assessed in 2-hour MCAO+24-hour reperfusion and 4-hour MCAO+24-hour reperfusion groups with or without NBO treatment.

Human Study I

Study Design

All procedures performed in studies involving human participants were in accordance with the Institutional Ethics Committee of Xuanwu Hospital, Capital Medical University. To investigate the changes of occludin and claudin-5 levels in human blood samples
after AIS, we recruited 8 patients with AIS who were admitted within 4.5 hours after stroke onset at Xuanwu Hospital between June 15, 2016, and November 23, 2016. In addition, 8 healthy people were randomly chosen from the physical examination population age between 40 and 69 years from the check center in Xuanwu Hospital on December 15, 2016.

**Inclusion and Exclusion Criteria of Patients With AIS**
The inclusion criteria were (1) age ≥18, presenting <4.5 hours after witnessed symptom onset; (2) eligible for intravenous thrombolysis; (3) National Institutes of Health Stroke Scale (NIHSS) score <25; (4) pre-admission modified Rankin Scale score <1; and (5) AIS was confirmed by computed tomography or magnetic resonance imaging following the day. The exclusion criteria were (1) active chronic obstructive pulmonary disease, (2) >3-L/min oxygen required to maintain peripheral arterial oxygen saturation >95% per stroke management guidelines; (3) rapidly improving neurological deficits; (4) medically unstable; (5) pregnancy; and (6) inability to obtain informed consent. All enrolled patients with AIS received intravenous tPA thrombolytic therapy and standard clinical treatment (anticoagulant and antiplatelet). The general conditions of patients are shown in Table I in the online-only Data Supplement.

**Measurement of Blood Sample From Patients With AIS and Healthy People**
Blood samples (4 mL) from 8 patients with AIS and 8 healthy subjects were collected to measure blood occludin and claudin-5 at the following time points: at admission, 24 hours (20–28 hours), and 72 hours (68–76 hours). Sera (100 µL) were then separated to measure the levels of occludin and claudin-5 with commercially available ELISA kits for human samples (occludin: USCN, China; claudin-5: USCN, China).

**Human Study II**

**Study Design**
To investigate the changes of claudin-5 and occludin in AIS patients with or without NBO treatment, another 18 patients with AIS between November 23, 2016, and January 10, 2017, were recruited and randomly divided into NBO and normoxia group. The criteria for inclusion and exclusion and the methods to measure blood sample were the same as in human study I.

**NBO Treatment in Patients With AIS**
As soon as diagnosis of AIS was made based on clinical symptoms and computed tomography, patients in NBO group were immediately given oxygen inhalation for 4 hours by oxygen facemask at a flow rate of 10 L/min. Normoxia group inhaled room air.

**Measurement of Neurological Functions by NIHSS Scores**
NIHSS scores were measured to determine the relationship between the outcome and biomarker levels after NBO treatment. NIHSS scores were recorded at admission, 24 hours (20–28 hours), 72 hours (68–76 hours), and 1 week (range, 6–8 days).

**Statistical Analysis**
The data are presented as means±SEM. Statistical analysis was performed using ANOVA and χ^2. Repeated measures ANOVAs were used to analyze differences in related variables. A value of *P*<0.05 was considered statistically significant.

**Results**

**NBO-Treated Rats Significantly Slowed Ischemic BBB Damage**
We compared the extent of BBB damage with or without NBO treatment after 2- or 4-hour ischemia followed by 2-hour reperfusion. EB extravasation into brain tissue was shown in Figure 1. As expected, EB contents in nonischemic hemispheric tissue were low in all groups. Cerebral ischemia/reperfusion considerably increased EB leakage into the ischemic hemisphere after 2-hour ischemia/reperfusion in normoxic group, and EB leakage was further elevated when ischemia duration was increased to 4 hours. Importantly, NBO treatment significantly reduced EB leakage in 4-hour ischemia/reperfusion rats when compared with the normoxic rats (Figure 1). These results indicate that NBO treatment can attenuate BBB disruption after cerebral ischemia/reperfusion.

**NBO Reduces the Loss of TJPs in Microvessels in Cerebral Ischemia Rats**
To investigate the kinetics of TJPs loss during cerebral ischemia/reperfusion under NBO or normoxia condition, we extracted cerebral microvessels from ischemic hemispheric tissue to determine the levels of occludin (Figure 2A) and claudin-5 (Figure 2B) in the isolated microvessels. Western blot for occludin showed that rats in sham group have high level of occludin. Although 2 hours of ischemia did not induce detectable loss of occludin proteins in ischemic cerebral microvessels, there were significant reductions observed in 4-hour MCAO and 4-hour MCAO plus 2-hour reperfusion. Of note, NBO treatment significantly prevented occludin in microvessels from degradation both in 4-hour MCAO and...
in 4-hour MCAO with 2-hour reperfusion. Similar results were observed for claudin-5 in microvessels (Figure 2B). These results indicate NBO can slow the loss of occludin and claudin-5 proteins in ischemic microvessels during cerebral ischemia/reperfusion.

**NBO Reduces the Levels of Occludin in Blood Sample of Rats**

Blood occludin in sham rats was constantly at low level at all 5 time points, showing that the microvessels were healthy and intact. The levels of blood occludin were doubled after 4-hour cerebral ischemia and then slightly fell after reperfusion but still remained significant higher than that of sham group (Figure 3A). It is noteworthy that NBO could prevent ischemia-induced occludin increase in blood, keeping the level close to the baseline of sham group. Unlike occludin, we did not observe significant changes of blood claudin-5 in response to different cerebral ischemia durations, neither did NBO significantly affect blood claudin-5 level during ischemia/reperfusion (Figure 3B). These results indicate that blood occludin levels mirrored the extent of BBB injury during ischemia/reperfusion, and NBO could protect BBB by suppressing occludin degradation from the microvessels that leads to reduced occludin in the blood circulation.

**Effects of NBO on Neurological Scores and Mortality in Rats With Ischemic Stroke**

As a consequence of protecting the integrity of BBB, we expect that NBO could improve the functional outcomes in animals subjected to ischemia/reperfusion. Neurological functions in NBO or normoxia groups were measured after 2-hour ischemia, 4-hour ischemia, and 4-hour ischemia+2-hour reperfusion. There was no significant difference between NBO and control groups after 2-hour ischemia. However, NBO treatment improved neurological scores at 4-hour MCAO and 4-hour ischemia+2-hour reperfusion, compared with rats in the normoxia group (Figure 4A).

![Figure 2. Normobaric hyperoxia (NBO) reduced the loss of tight junction proteins in isolated microvessels. A, Representative Western blots and quantitative analysis of occludin in isolated microvessels following indicated ischemia/reperfusion (I/R) time. B, Representative Western blots and the quantitative analysis of the level of claudin-5 in isolated microvessels. N=4. *P<0.05 vs sham group rats; #P<0.05 vs normoxic rats with same duration of I/R. Data are expressed as means±SEM.](image)

![Figure 3. Changes of blood tight junction proteins, and effects of normobaric hyperoxia (NBO) after middle cerebral artery occlusion (MCAO). A, Levels of occludin in blood of ischemic rats. Four-hour MCAO induced a significant increase of blood occludin and remained high in subsequent 5-minute and 2-hour reperfusion; NBO treatment reduced blood occludin. N=6. *P<0.05 vs I-0 h in sham group, #P<0.05 vs the normoxia rats with the same ischemic duration. B, Levels of claudin-5 in blood. No significant difference of blood claudin-5 level was observed among 3 groups. Data were presented as means±SEM.](image)
Mortality in both groups was determined at the end of 24-hour reperfusion after 2- or 4-hour ischemia (Figure 4B). Four-hour ischemia in normoxia resulted in a higher mortality to 62.5% (5/8) than 2-hour ischemia at 12.5% (1/8). NBO significantly decreased the mortality in 4-hour ischemia/reperfusion rats to 11.1% (1/9). These results suggest that NBO can improve the outcome of ischemia/reperfusion rats.

The Change of Blood Occludin and Claudin-5 Levels in Patients With AIS

Blood samples from 8 patients with AIS were collected when they arrived at hospital, as well as 24 hours (range, 20–28 hours) and 3 days (range, 66–78 hours) since symptoms appeared. Eight healthy people were chosen from check center for physical examination. There were no significant differences in mean age, sex, and underlying conditions (such as diabetes mellitus and myocardial infarction) in these 2 groups, but there was a significant difference in history of hypertension (Table I in the online-only Data Supplement). ELISA assay showed that the basal level of occludin was relatively low in serum of healthy volunteers. However, blood occludin in patients with AIS significantly increased at admission (≈4.5 hours since symptoms appeared) and remained at high level ≤72 hours since stroke onset (Figure 5A). There was a trend of slight, but not statistically significant, increase in blood claudin-5 in patients with AIS after stroke (Figure 5B). These results provide direct clinical evidence that blood occludin increased in response to cerebral ischemia.

NBO Reduced Blood Occludin and Improved Neurological Functions in Patients With AIS

We further investigated whether NBO treatment can reduce blood occludin in patients with AIS. There was no intracranial hemorrhage, subarachnoid hemorrhage, or death occurred in either group when they were in hospital. There was no significant difference in the average age, sex, admission time, underlying conditions (such as diabetes mellitus, myocardial infarction, arterial fibrillation, and history of stroke), and NIHSS scores at admission (Table II in the online-only Data Supplement).

ELISA assay demonstrated that blood occludin was elevated during ischemia/reperfusion in both rats and human. The present study reports that blood occludin increased as a result of ischemia/reperfusion-induced BBB damage, not only in ischemic rats but also in AIS patients with intravenous tPA thrombolysis. Moreover, NBO could downregulate the level of blood occludin and significantly improve neurological functions at early phase of AIS, as reflected by the reduced occludin levels in blood and NIHSS scores.

Discussion

The present study reports that blood occludin increased as a result of ischemia/reperfusion-induced BBB damage, not only in ischemic rats but also in AIS patients with intravenous tPA thrombolysis. Moreover, NBO could downregulate the level of blood occludin and significantly improve neurological outcomes during ischemia/reperfusion in both rats and human.

To date, intravenous tPA thrombolysis is only approved by the United States Food and Drug Administration for treatment of ischemic stroke patients within a strict temporal window. This one-size-fits-all time window prevents stroke patients with a low risk of intracerebral hemorrhage from receiving the benefits of tPAs. Therefore, it is important to seek biomarkers, which could accurately evaluate the extent of BBB damage during cerebral ischemia/reperfusion, to guide tPA thrombolysis.
Some studies have reported about pretreatment biomarkers to guide stroke thrombolysis. However, to date, no reliable biomarkers are able to accurately predict the extent of BBB damage before tPA administration. Our recent study showed that elevated blood occludin level may be a promising biomarker for evaluating BBB during the period of ischemia in ischemic model of rats. In the present study, we investigated the level of blood occludin and claudin-5 during ischemia and reperfusion in both rats and human.

Blood occludin in rats significantly increased at 4-hour MCAO and remained at significantly higher level than sham group at 4-hour MCAO with 5-minute and 2-hour reperfusion. At the same time, occludin was lost from the microvessels isolated from ischemic brain tissue. On the contrary, there was no significant change in blood claudin-5 after cerebral ischemia/reperfusion. These data suggest that increased blood occludin may reflect the extent of BBB damage during ischemia/reperfusion in ischemic model of rats.

We also investigated the changes of blood occludin and claudin-5 in patients with AIS, who underwent intravenous tPA thrombolysis. We found that compared with healthy people, occludin was significantly increased in AIS patients’ blood samples, which were collected on their arrival at hospital. These data suggest that ischemia-induced BBB damages are detectable when patients with AIS arrived at hospital (within 4.5 hours after stroke) using blood occludin as a biomarker. Blood occludin in patients with AIS remained at a high level at 24 hours and 3 days, suggesting either that ischemia/reperfusion induced a sustained BBB damage process or that its clearance from blood is limited. Our findings imply that blood occludin is a promising, clinically relevant biomarker for evaluating the microvessel damage of individual patient and potentially guiding tPA thrombolysis.

We further examined whether ischemia/reperfusion-induced occludin degradation and its fragments in blood could be suppressed if given a BBB-protective intervention (ie, NBO). Many recent studies showed that NBO given during cerebral ischemia is vasoprotective in animals. NBO can inhibit matrix metalloproteinase-9 activity in the ischemic brain, thereby preventing occludin degradation, EB extravasation, and hemispheric swelling. In the present study, we demonstrate that NBO treatment effectively slowed the degradation of occludin on microvessels during ischemia/reperfusion and reduced blood occludin fragments in ischemic rats. Our data in patients with AIS confirm that NBO can suppress the level of occludin and its fragments in blood at 24 hours, 3 days, and 1 week after stroke. These data from patients with stroke suggest that NBO could reduce the level of blood occludin, which reflect the decreased BBB damage in these patients.

In this study, we observed NBO’s effects on neurological outcome in animals and in patients. Our results indicate that NBO treatment significantly improved neurological outcome and reduced mortality in the ischemic model of rats. Moreover, NBO can improve neurological function of patients with AIS, which is consistent with previous clinical pilot studies where NBO transiently improved stroke scale scores, reduced mortality and comorbidities in patients with severe AIS, and attenuated ischemic lesion growth. In the present study, we observed NBO’s neuroprotective effects in patients with AIS who underwent thrombolysis after admission, and these benefits persisted at 3 days and 1 week. These findings from a pilot study with limited number of patients provide an important basis for further examining the potential application of NBO for BBB protection in the clinic. Larger scale clinical studies are warranted to investigate the long-term effects and optimum duration of NBO in patients with thrombolysis.

In conclusion, the results from our study suggest that blood occludin has the potential to be a biomarker of BBB damage during both ischemia and reperfusion period for both animal and human stroke. More importantly, our findings indicate that NBO may be a clinically relevant approach to protect BBB by reducing occludin degradation from microvasculature, leading to improved neurological functions for both animal and human stroke. Consequently, NBO has the potential to serve as an efficacious adjuvant therapy for tPA thrombolysis in ischemic stroke in the clinic.

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Disclosures
None.

References


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Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2017/09/20/STROKEAHA.117.017713.DC1

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Detailed information on occludin antibodies for the ELISA assay

Both rat and human ELISA assay kits for occludin were purchased from USCN Life Science Inc., China. According to the information provided by the vendor, the specifics for the antibody for rats are: target sequences, Ala88–Arg269; sensitivity, 0.312ng/ml-20ng/ml; minimum, 0.112ng/ml; and specificity, 100%. The specifics for human kit are: target sequences, Ser370–Thr522; sensitivity, 0.156ng/ml-10ng/ml; minimum, 0.055ng/ml; and specificity, 100%. The standard calibration curves for rat and human occludin are shown below:
Evan’s blue assay to measure BBB damage

Evan’s blue dye binds to albumin in plasma and the complex enter into brain tissue through compromised BBB. Quantitative analysis of Evan’s blue dye in brain tissue is commonly used to measure the extent of BBB injury in animal models (Stroke, 2015, 46: 1344-1351; J Neurosci. 2013, 33: 19579-89). Evan’s blue dye (EB, 2% wt/vol in PBS, 3mL/kg) (Sigma, USA) was administered (I.V.) via tail vein at the onset of reperfusion. At the end of 2 h reperfusion, the rats were transcardially perfused with 250 mL cold PBS. The brain was then removed, and hemispheric tissues were homogenized in 1 ml 50% trichloroacetic acid. The contents of the dye were assessed by measuring the fluorescence intensity using Odyssey Infrared Imaging System (emission wavelength of 680nm). The total Evan’s blue content (ng/g) in each sample was calculated according to the external standard curve. The difference of dye contents in ischemic hemispheric tissue between Normoxia and NBO groups reflected the extent of BBB damage. The standard curve for Evan’s blue assay is shown below:
### Supplemental Table I. Baseline Data for 8 AIS patients and 8 healthy volunteers (Human Study I)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>AIS patients (n=8)</th>
<th>Healthy volunteers (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y (means, range)</td>
<td>64.6(33-77)</td>
<td>50.6(40-69)</td>
</tr>
<tr>
<td>Female</td>
<td>3(37.5%)</td>
<td>4(50%)</td>
</tr>
<tr>
<td>Onset time</td>
<td>3.5(1-4.5)</td>
<td>N/A</td>
</tr>
<tr>
<td>Prior medical history</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior stroke</td>
<td>2(25.0%)</td>
<td>0(0.0%)</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>0(0.0%)</td>
<td>0(0.0%)</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>2(25.0%)</td>
<td>0(0.0%)</td>
</tr>
<tr>
<td>Hypertension*</td>
<td>5(62.5%)</td>
<td>2(25.0%)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>2(25.0%)</td>
<td>1(12.5%)</td>
</tr>
<tr>
<td>Malignancy</td>
<td>0(0.0%)</td>
<td>0(0.0%)</td>
</tr>
</tbody>
</table>

*P<0.05 AIS patients vs Healthy volunteers
**Supplemental Table II. Baseline data for stroke patients in treatment and control groups (Human Study II)**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>NBO group (n=9)</th>
<th>Controls group (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y (mean, range)</td>
<td>61.6(51-75)</td>
<td>58.0(47-70)</td>
</tr>
<tr>
<td>Female</td>
<td>3(33.3%)</td>
<td>2(28.6%)</td>
</tr>
<tr>
<td>Onset time</td>
<td>3.2(1-4.5)</td>
<td>3.0(1.2-4.5)</td>
</tr>
<tr>
<td>Admission NIHSS</td>
<td>12.0(5-20)</td>
<td>12.3(7-18)</td>
</tr>
</tbody>
</table>

Prior medical history

<p>| Prior stroke                        | 1(11.1%)        | 2(22.2%)             |
| Myocardial infarction               | 1(11.1%)        | 0(0.0%)              |
| Atrial fibrillation                 | 1(11.1%)        | 0(0.0%)              |
| Hypertension                        | 6(66.7%)        | 7(77.8%)             |
| Diabetes                            | 5(55.6%)        | 3(33.3%)             |
| Malignancy                          | 0(0%)           | 0(0%)                |</p>
<table>
<thead>
<tr>
<th>Methodological and Reporting Aspects</th>
<th>Description of Procedures</th>
</tr>
</thead>
</table>
| Experimental groups and study timeline | ✓ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study.  
✓ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated.  
✓ An overall study timeline is provided. |
| Inclusion and exclusion criteria | ✓ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article. |
| Randomization | ✓ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided.  
✓ Type and methods of randomization have been described.  
✓ Methods used for allocation concealment have been reported. |
| Blinding | ✓ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible.  
✓ Blinding procedures have been described with regard to masking of group assignment during outcome assessment. |
| Sample size and power calculations | ✓ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided. |
| Data reporting and statistical methods | ✓ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups. |
| Experimental details, ethics, and funding statements | √ Baseline data on assessed outcome(s) for all experimental groups have been reported. √ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms. √ Statistical methods used have been reported. √ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures. | √ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described. √ Different sex animals have been used. If not, the reason/justification is provided. √ Statements on approval by ethics boards and ethical conduct of studies have been provided. √ Statements on funding and conflicts of interests have been provided. |