Hyperbaric Oxygen-Induced Attenuation of Hemorrhagic Transformation After Experimental Focal Transient Cerebral Ischemia

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Background and Purpose—An increased risk of hemorrhagic transformation is a major factor limiting the use of tissue plasminogen activator for stroke. Increased hemorrhagic transformation is also found in animals undergoing transient focal cerebral ischemia with hyperglycemia; this study examined whether hyperbaric oxygen (HBO) could reduce such hemorrhagic transformation in a rat model.

Methods—Rats received an injection of 50% glucose (6 mL/kg intraperitoneally) and had a middle cerebral artery occlusion 10 minutes later. Rats were treated with HBO (3 ATA for 1 hour) 30 minutes after middle cerebral artery occlusion. Control rats received normobaric room air. Rats underwent reperfusion 2 hours after middle cerebral artery occlusion. Blood–brain barrier permeability (Evans blue), hemorrhagic transformation (hemoglobin content), brain edema, infarct volume, and mortality were measured.

Results—HBO treatment reduced Evans blue leakage in the ipsilateral hemisphere (28.4 ± 3.5 versus 71.8 ± 13.1 μg/g in control group, \( P < 0.01 \)) 2 hours after reperfusion and hemorrhagic transformation (0.13 ± 0.13 versus 0.31 ± 0.28 mg hemoglobin in the control group, \( P < 0.05 \)) 22 hours later. Mortality was less in the HBO group (4% versus 27% in controls, \( P < 0.05 \)). Mean infarct volume and swelling in the caudate were also less in HBO-treated rats (\( P < 0.05 \)), but HBO failed to reduce brain water content in the ipsilateral hemisphere (\( P > 0.05 \)).

Conclusions—Early intraischemic HBO treatment reduces the blood–brain barrier disruption, hemorrhagic transformation, and mortality after focal cerebral ischemia suggesting that HBO could be used to reduce hemorrhagic conversion in patients with stroke. (Stroke. 2007;38:1362-1367.)

Key Words: blood–brain barrier permeability ■ brain edema ■ cerebral ischemia ■ hemorrhagic transformation ■ hyperbaric oxygen

There is currently no effective treatment to limit the occurrence or effect of hemorrhagic transformation after a stroke. Such hemorrhagic transformation is a major factor limiting the use of tissue plasminogen activator (tPA) to reduce ischemic brain damage in patients.¹² The only U.S. Food and Drug Administration-approved therapy for ischemic stroke, tPA is associated with an approximately 5% to 6% risk of symptomatic intracerebral hemorrhage when given within 3 hours of stroke onset¹ and an 8% to 9% risk of symptomatic intracerebral hemorrhage when tPA is given 3 to 6 hours after stroke onset as compared with a 1% to 2% risk in placebo-treated patients.⁴ The rate of tPA-associated intracerebral hemorrhage (ICH) has both direct and indirect adverse consequences for patients with stroke. First, ICH after treatment with tPA is associated with dramatically worsened clinical outcomes. The majority of patients with symptomatic hemorrhage later die.⁵ Intracerebral hemorrhage causes brain damage and death through a number of mechanisms.⁶ In addition to these direct consequences, fear of causing ICH has been shown to be the reason many physicians refuse to treat eligible patients with tPA despite its overall efficacy.⁷⁸

Hyperglycemia has a major impact on posts ischemic hemorrhagic transformation. Thus, De Courten-Myers et al reported that acute hyperglycemia dramatically increases brain infarct and hemorrhagic conversion in a cat middle cerebral artery occlusion (MCAO) with reperfusion model.⁹ A similar effect on hemorrhagic conversion has been found in rats undergoing MCAO with reperfusion.¹⁰ Most importantly, human data shows that hyperglycemia is a very strong predictor of ICH in patients undergoing tPA therapy.¹¹ Hyperglycemia-enhanced damage in stroke may be linked to increased inflammatory activity, oxidative stress, and free radical production.¹¹–¹³
Hyperbaric oxygen (HBO) treatment is neuroprotective in many experimental studies, including cerebral ischemia, subarachnoid hemorrhage, and brain trauma. It has been reported that early use of HBO reduces blood–brain barrier (BBB) disruption, brain edema, and infarct volume and improves functional outcome after experimental cerebral ischemia. However, it is not clear whether HBO can reduce hemorrhagic transformation after cerebral ischemia.

The present study investigated the effects of HBO on hemorrhagic conversion, BBB permeability, brain edema, brain infarct volume, and neurologic deficit after focal cerebral ischemia with reperfusion in a hyperglycemic rat model.

Materials and Methods

Animal Preparation and Middle Cerebral Artery Occlusion

Animal study protocols were approved by the University of Michigan Committee on the Use and Care of Animals. A total of 81 male Sprague-Dawley rats (Charles River Laboratories; Portage, Mich) weighing 275 to 325 g were used in this study. Rats were allowed free access to food and water before surgery. Anesthesia was induced by inhalation of 5% isoflurane in a nitrous oxide/oxygen mixture (70/30) and maintained by 1.5% isoflurane administered through a facemask. Rectal temperature was maintained at 37.5°C with use of a feedback-controlled heating pad. Rats were injected with 50% glucose (6 mL/kg) intraperitoneally to induce acute hyperglycemia.

Middle cerebral artery occlusion was induced using the filament model as previously described with some modifications. Briefly, under an operating microscope, the left common, external, and internal carotid arteries were dissected from connective tissue through a midline neck incision. After the branches of the external carotid artery were dissected and coagulated, the external carotid artery was cut, leaving a stump as long as possible attached to the common carotid artery. The pterygopalatine artery of the internal carotid artery was separated and ligated. Then blood was obtained from the tail artery for analysis of blood pH, PaO2, PaCO2, and blood glucose. We measured blood gases before MCAO to make sure the physiological conditions were the same in HBO-treated and control animals at baseline. We did not measure peak blood oxygen content resulting from HBO, which is technically difficult when animals are conscious and isolated in the chamber. It has previously been shown that peak arterial PO2 resulting from HBO treatment at 3 ATA is over 1000 mm Hg (higher than the range accurately measured by most blood gas devices) and this results in an increase in total arterial oxygen content of approximately 20% in anesthetized animals. A 23-mm segment of 3-0 nylon monofilament suture with the tip rounded by a flame was inserted into the stump of the left common carotid artery and advanced into the internal carotid artery approximately 19 to 20 mm from the bifurcation to occlude the origin of the MCA.

Two hours after MCAO introduction, animals were reanesthetized and the filament was removed. The rats were allowed to awaken and recover with free access to food and water.

Laser Doppler Flowmetry

Cerebral blood flow was examined in the core area of MCA territory by laser Doppler before and 10 minutes after MCAO and after 10 minutes of reperfusion. Data are presented as a percentage of baseline.

Experimental Groups

There were five parts in this study. All rats had MCAO with acute hyperglycemia. Rats were treated with HBO (3 ATA for 1 hour) 30 minutes after MCAO. Control rats received normobaric room air. In the first part, the rats (n=6 per group) were killed 2 hours after reperfusion for Evans blue content measurement. In the second part, rat brains were sampled (n=10 to 12) for hemoglobin content determination 24 hours after MCAO. Rats (n=10 to 12) in the third part were used for histologic examination, including the measurement of infarct volume and brain swelling, 24 hours after MCAO. In the fourth part, brain water content (n=5 to 7) was determined 24 hours after MCAO. In the last part, neurologic deficits (n=6 to 7) were measured at 24 hours and cerebral blood flow was examined. In animals assigned to 24-hour survival, the effect of HBO therapy on overall mortality was also observed.

Hyperbaric Oxygen Administration

Animals in the HBO treatment group were placed in a small rodent HBO chamber (Marine Dynamics Corp; Long Beach, CA) 15 minutes after the onset of ischemia. HBO-treated animals were pressurized over 15 minutes to a plateau pressure of 3 ATA with 100% oxygen supplied continuously and maintained for 60 minutes. Decompression was then carried out over 25 to 30 minutes. Control animals were also transferred into the HBO chamber but received normobaric room air.

Measurement of Evans Blue

The integrity of the BBB was investigated by measuring the extravasation of Evans blue in HBO-treated animals and controls (n=6 each). Evans blue dye (2% in saline, 4 mL/kg) was given intravenously 2 hours after MCAO, just after the removal of the intraluminal filament. Two hours after Evans blue injection, the chest wall was opened under lethal anesthesia and the animals were perfused with 0.1 mol/L phosphate-buffered saline through the left ventricle to remove the intravascular localized dye until colorless perfusion fluid was obtained from the right atrium. After decapsulation, the brain was removed and dissected into left and right hemispheres and each hemisphere was weighed. Brain samples were then placed in 3 mL 50% trichloroacetic acid solution and then homogenized and centrifuged (10,000 rpm for 20 minutes). The supernatant was measured at 610 nm for absorbance using a spectrophotometer (Ultrospec 3; Pharmacia LKB). The tissue content of Evans blue was quantified from a linear standard curve and was expressed as micrograms per gram of brain tissue.

Spectrophotometric Assay of Hemoglobin

Hemorrhagic transformation was quantified with spectrophotometric assay of brain hemoglobin content. At 24 hours after MCAO (22 hours after reperfusion), the animals were perfused transcardially with 0.1 mol/L phosphate-buffered saline under deep anesthesia until the outflow fluid from the right atrium was colorless. The brain was rapidly removed and dissected into two parts, the left hemisphere and the right hemisphere. The hemispheric brain tissue was then homogenized in 0.1 mol/L phosphate-buffered saline followed by 30-minute centrifugation (15,000 rpm). The 200 μL reagent (QuantChrom Hemoglobin Assay Kit; BioAssay Systems) was added to 50 μL supernatant. After 15 minutes, optical density was measured at 400 nm with a spectrophotometer (Ultrospec 3; Pharmacia LKB). The total hemispheric hemoglobin content was expressed as milligrams per hemisphere.

Histopathology

Twenty-four hours after MCAO, the animals were reanesthetized and perfused intracardially with 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (pH 7.4). Brains were removed and kept in 4% paraformaldehyde for 6 hours and then immersed in 25% sucrose for 3 to 4 days at 4°C. The brains were embedded in the mixture of 25% sucrose and optimal cutting temperature compound (Sakura Finetek) and 20-μm-thick coronal frozen sections were made on a cryostat. After disposing the 2-mm anterior part of the forebrain, slices for every 2-mm-thick interval distance were stained with hematoxylin and eosin. In total, five slices of each brain were stained and analyzed. To quantify infarct volume, the areas of infarction at five coronal levels throughout the brain were identified and the infarct and hemispheric volumes were measured with National Institutes of Health Image by an investigator blind to the treatments (M.K.). The extent of brain swelling was determined by subtracting the contralateral hemispheric volume from the ipsilateral. Infarct volume and swelling were measured and analyzed by whole hemi-
sphere and by region (cortex and basal ganglia). To avoid artifact in volume measurement from brain edema within the infarct, infarct volume was calculated by measuring and subtracting the volume of the noninfarcted ipsilateral hemisphere from the volume of the contralateral hemisphere.23

Brain Water Content

Twenty-four hours after MCAO, animals were anesthetized and decapitated as described in our previous study.24 The brains were removed and dissected into three parts: the cerebellum and left and right hemispheres. Brain samples were immediately weighed on an electronic balance (model AE 100; Mettler Instrument) to obtain wet weight. Brain samples were then dried in a gravity oven (Blue M. Electric Co) at 100°C for 48 hours to obtain the dry weight. Brain water content was then calculated as (wet weight – dry weight) x 100/ wet weight.

Neurologic Deficits

Twenty-four hours after MCAO, a neurologic examination was performed by a blinded investigator as previously described with modifications.25 Briefly, the scores were 0 (no apparent deficits), 1 (contralateral forelimb flexion when suspended by the tail), 2 (decreased grip of the contralateral forelimb while tail pulled), 3 (spontaneous movement in all directions; contralateral circling only if pulled by the tail), 4 (spontaneous contralateral circling), and 5 (death after recovery from the anesthesia). Animals that showed the features of the higher scores also showed all the features of the lower grades.

Statistical Analysis

Data are expressed as mean±SD. Statistical significance was analyzed by two-tailed Student t test and Mann–Whitney U tests for continuous variables and by $\chi^2$ test for mortality. A probability value of <0.05 was considered statistically significant. To compare the ratio of Evans blue content with infarct size in HBO-treated and control animals, data underwent log transformation before a t test.

Results

Physiological variables were measured immediately before the introduction of MCAO. The levels of pH, Po2, and PCO2 were controlled in the normal range (Table). Blood glucose levels just before MCAO were not significantly different between HBO- and control-treated rats (476±38 versus 479±44 mg/dL, respectively, $P>0.05$). There was also no significant difference in cerebral blood flow between the HBO-treated group and the control group either after MCAO (10.1±6.8% of baseline versus 10.5±4.5% in controls, $P>0.05$) or after reperfusion (71.1±13.5% versus 89.0±28.3% in controls, $P>0.05$).

There was a marked increase in Evans blue content in the ipsilateral hemisphere as compared with the contralateral hemisphere in control animals 2 hours after 2-hour MCAO (71.8±32.1 versus 4.4±1.5 µg/g, $P<0.01$). HBO markedly reduced the extravasation of Evans blue in the ipsilateral hemispheres of treated animals as compared with controls (28.4±8.6 versus 71.8±32.1 µg/g in the control group, $P<0.01$; Figure 1).

Spectrophotometric measurement of brain hemoglobin showed that hemoglobin contents in the ipsilateral hemisphere 24 hours after MCAO were significantly higher than those in the contralateral side (653±261 versus 342±80 µg, $P<0.01$) in the vehicle-treated animals. HBO therapy reduced hemoglobin contents compared with those in the control group ($P<0.05$, Figure 2). Hemorrhagic transformation could be detected microscopically in the ipsilateral hemisphere of all animals, and it was more severe in control-compared with HBO-treated rats (Figure 3) in accordance with results of the hemoglobin assay. It should be noted that hemorrhagic transformation in most animals was microscopic hemorrhage. Only one animal in the control group, which died 24 hours after MCAO, developed a macroscopic hemorrhage.

Mean total infarct volumes 24 hours after MCAO were lower in HBO-treated animals than in control animals, but the difference did not attain statistical significance (110.8±63.4 versus 170.1±67.9 mm³, $P=0.08$). Although the reduction in cortical infarct volume with HBO was also not significant, HBO treatment did reduce infarct volume in the basal ganglia (49.0±24.5 versus 78.0±22.8 mm³ in the control, $P<0.05$; Figure 4).
To examine whether the effect of HBO treatment on BBB disruption (Evan blue content) was specific or the result of the reduction in infarct size, the ratio of Evans blue content to infarct volume was compared in the HBO and control groups. The Evans blue content per cubic millimeter of infarct was not quite significantly lower in the HBO-treated animals ($P = 0.056$, two-tailed).

Brain swelling occurred in the ipsilateral hemisphere after MCAO. Mean hemisphere volumes in the ipsilateral side were larger than that in the contralateral side in both groups. The total hemispheric swelling between groups was not significantly different ($63.9 \pm 32.7$ mm$^3$ in HBO-treated group versus $89.5 \pm 26.7$ mm$^3$ in controls, $P = 0.07$), but the ipsilateral basal ganglia swelling was reduced in the HBO-treated group ($25.9 \pm 11.8$ versus $38.9 \pm 9.7$ mm$^3$ in controls, $P < 0.05$).

Brain water content was significantly increased 24 hours after MCAO in the ipsilateral hemisphere. However, HBO treatment did not reduce brain water content in the ipsilateral hemisphere ($81.4 \pm 0.7\%$ versus $81.9 \pm 1.2\%$ in controls, $n = 5$, $P < 0.05$).

HBO did not improve gross neurologic deficits ($3.7 \pm 0.8$ versus $3.7 \pm 1.5$ in controls, $P > 0.05$), but it did reduce mortality rate at 24 hours in this MCAO model (4% versus 27% in controls, $n = 26$ to 33, $P < 0.05$, $\chi^2$ test). Of the control rats that died, 2, 2, 2, and 3 rats were from the hemoglobin, histology, edema, and behavior experimental groups, respectively. From the HBO rats, one died from the behavior group.

**Discussion**

Accumulating evidence indicates that HBO may have beneficial effects in stroke. Experimental studies have shown that early use of HBO may be neuroprotective in neonatal hypoxia–ischemia and focal and global cerebral ischemia.\textsuperscript{14} We have demonstrated that HBO can also attenuate hemorrhagic transformation in a rat model of focal cerebral ischemia. This effect was accompanied by reductions in BBB permeability, infarct volume, and brain swelling.

We studied hyperglycemia-induced hemorrhagic conversion in this ischemic model for two main reasons. First and foremost, because hyperglycemia is strongly associated with hemorrhagic transformation and tPA-associated ICH, an understanding of how hyperglycemia-induced bleeding might be prevented is clinically relevant.\textsuperscript{11} The second reason is the model produces a consistent hemorrhagic transformation after 2 hours of MCAO with reperfusion. The alternative embolic model with tPA-induced reperfusion is less consis-

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**Figure 3.** Hemorrhagic conversion in control (A, B, and C) and hyperbaric oxygen-treated (D, E, and F) rats 24 hours after middle cerebral artery occlusion. Scale bar=100 μm.

**Figure 4.** Brain infarct volume at 24 hours after middle cerebral artery occlusion with reperfusion in control and hyperbaric oxygen-treated rats. Values are mean±SD, $n = 10$. *$P < 0.05$ versus control.
tent in both frequency and location of hemorrhage. That acute hyperglycemia causes greater hypoxic–ischemic brain damage was reported 30 years ago. In cat and rat models of MCAO with reperfusion, hyperglycemia induces hemorrhagic conversion. Although the precise mechanisms that enhance hemorrhagic infarction are unknown, acidosis may be involved in hyperglycemia-induced hemorrhagic conversion. Recent studies indicate that hyperglycemia can increase production of matrix metalloproteinases, reactive oxygen species, and proinflammatory cytokines that might result in vascular damage and lead to hemorrhage. The effects of hyperglycemia on stroke are particularly detrimental during transient rather than permanent ischemia.

The major aim of this study was to examine whether HBO therapy during ischemia would reduce hemorrhagic transformation after focal cerebral ischemia with reperfusion and indeed HBO did reduce such hemorrhage as assessed by brain hemoglobin content and microscopy. Although the mechanism of HBO-induced attenuation of hemorrhagic transformation is not well understood, our results suggest that HBO reduces hemorrhagic transformation in the rat model of cerebral ischemia by reducing BBB disruption. The BBB is a physiological barrier to the movement of many molecules between blood and the brain. BBB disruption after cerebral contributes to secondary brain injury after cerebral ischemia and hemorrhagic conversion. This effect of HBO on BBB disruption described in this study is supported by recent findings by Veltkamp et al, who found that HBO therapy reduces postischemic BBB damage in rats and mice. HBO reduces basal lamina degradation and upregulation of plasma matrix metalloproteinase-9 after experimental cerebral ischemia, which are associated with hemorrhagic transformation. HBO has also been reported to decrease inflammation after cerebral ischemia, which may reduce vascular damage.

In addition to the effects of HBO on BBB permeability, the present results also demonstrate that HBO reduces brain infarction in hyperglycemic animals. It has been reported by several groups that HBO therapy can decrease infarct volume. It is not clear whether less hemorrhagic conversion after HBO treatment results from this smaller brain infarct. However, the hemoglobin and Evans blue content in the HBO group was 50% to 60%, respectively, less than that in controls, whereas the infarct volume was only approximately 35% less. This suggests that this reduction in infarct volume with HBO may not entirely account for the reduction in BBB disruption.

Although we cannot prove causality, the observation that the protective effects of HBO are maintained or increased (rather than eliminated) in this model of amplified BBB disruption are consistent with protection of the BBB as a mechanism relevant to this treatment. Brain edema contributes to secondary brain injury after stroke. The predominant form of edema after cerebral ischemia is cytotoxic, in which there is swelling of parenchymal cells, but there is also vasogenic edema attributable to BBB disruption. In the present study, we found that HBO reduced BBB disruption and swelling of the basal ganglia significantly. However, HBO did not affect cortical swelling significantly, and this limited local effect on swelling may explain why there was not an effect on total hemispheric water content after MCAO.

Another important finding is that HBO reduces mortality in this hyperglycemic stroke model. The death rate in the control group was approximately 27%, whereas it was 4% among the HBO-treated rats. This lower mortality may result from the smaller infarct, less hemorrhagic transformation, and less BBB disruption in the HBO-treated group. An additional possibility is that HBO may reduce hemorrhage-induced tissue injury, although a causal relationship is not established between mortality and hemorrhagic transformation. Intracerebral hemorrhage causes brain injury by a variety of mechanisms, and the effect of HBO on such mechanisms deserves further investigation.

In the current study, we did not find that HBO reduced neurologic deficits at 24 hours. It is still unclear why HBO did not improve function outcomes, but the behavioral tests used in this study may be not sensitive enough to differentiate the degree of brain injury in stroke models with severe brain damage such as the ischemia model with hyperglycemia.

The current model does not immediately translate into clinical practice because the treatment time used is short and administration of tPA rather than hyperglycemia is the usual precipitant. Our purpose was to assess the effects of optimal HBO therapy in a model in which postischemic BBB degradation is deliberately amplified. Because the putative effects of HBO on preservation of the extracellular matrix and endothelium are not specific to the injury caused by tPA, we feel that a generalized model of BBB breakdown and HBO is of interest. Although frank parenchymal hematomas are far more common in patients with stroke treated with tPA, loss of the BBB with either microhemorrhage or malignant vasogenic edema is common in patients with stroke regardless of treatment.

In conclusion, HBO reduces hemorrhagic conversion, BBB leakage, brain infarct, and mortality in a rat model of focal cerebral ischemia suggesting early HBO treatment may be useful for patients with stroke.

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Disclosures
None.

References
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