Original Contribution

Novel Hemoglobin-Based Oxygen Carrier Bound With Albumin Shows Neuroprotection With Possible Antioxidant Effects

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Background and Purpose—A hemoglobin-albumin cluster, 1 core of hemoglobin covalently bound with 3 shell albumins, designated as HemoAct was developed as a hemoglobin-based oxygen carrier. We aim to investigate neuroprotection by HemoAct in transient cerebral ischemia and elucidate its underlying mechanisms.

Methods—Male rats were subjected to 2-hour transient middle cerebral artery occlusion and were then administered HemoAct transarterially at the onset of reperfusion. Neurological and pathological findings were examined after 24 hours of reperfusion to identify neuroprotection by HemoAct. Intermittent measurements of cortical blood flow and oxygen content were performed, and a histopathologic analysis was conducted on rats during the early phase of reperfusion to assess the therapeutic mechanism of HemoAct. In addition, the antioxidant effects of HemoAct were examined in hypoxia/reoxygenation-treated rat brain microvascular endothelial cells.

Results—Neurological deterioration, infarct and edema development, and the activation of MMP-9 (matrix metalloprotease-9) and lipid peroxidation after 24 hours of reperfusion were significantly ameliorated by the HemoAct treatment. Reductions in blood flow and tissue partial oxygen pressure in the cortical penumbra after 6 hours of reperfusion were significantly ameliorated by the HemoAct treatment. The histopathologic analysis of the cortical penumbra revealed that HemoAct in HemoAct-treated rats showed superior microvascular perfusion with the mitigation of microvascular narrowing changes than autologous erythrocytes in nontreated rats. Although HemoAct extravasated into the ischemic core with serum protein, it did not induce an increase in serum extravasation or reactive oxygen species production in the ischemic core. In vitro experiments with rat brain microvascular endothelial cells revealed that HemoAct significantly suppressed cellular reactive oxygen species production in hypoxia/reoxygenation-treated cells, similar to albumin.

Conclusions—HemoAct exerted robust neuroprotection in transient cerebral ischemia. Superior microvascular perfusion with an oxygen delivery capability and possible antioxidant effects appear to be the underlying neuroprotective mechanisms.

Visual Overview—An online visual overview is available for this article. (***Stroke*. 2018;49:00-00. DOI: 10.1161/STROKEAHA.118.021467.)**

Key Words: antioxidant ■ brain ischemia ■ hemoglobin ■ neuroprotection ■ reperfusion

Because several randomized controlled trials on endovascular thrombectomy for acute ischemic stroke provided clear evidence of therapeutic efficacy,[1–4] therapeutic approaches to ischemic stroke have markedly changed. Endovascular thrombectomy achieved higher rates of angiographically demonstrated revascularization and provided better functional outcomes.[5] Although high recanalization rates are favorable for tissue salvage, delayed recanalization after severe ischemia contributes to tissue injury based on microvascular perfusion disorders. Severe neurological disorders and poor outcomes because of ischemia/reperfusion (I/R) injury have increased the need for neuroprotective therapeutic strategies against I/R injury.

Among various neuroprotective therapeutic strategies, artificial oxygen carriers, mainly hemoglobin-based oxygen carriers (HBOCs), have been used in anticipation of improvements in microvascular perfusion, more efficient oxygen delivery, and increases in collateral flow.[6–8] We previously demonstrated that liposome-encapsulated Hb, a cellular-type HBOC, reduced I/R injury in a rat transient middle cerebral artery occlusion

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(tMCAO) model.[9,10] In the present study, we used a novel cell-free HBOC, 1 core of Hb covalently bound with 3 human serum albumin molecules, designated as HemoAct.[11] Because HemoAct is covered with albumin shells, its surface net charge becomes negative and induces electrostatic repulsion against the endothelial surface, resulting in suppressed leakage through the endothelium. This property prevents marked elevations in blood pressure and promotes a long period of blood retention.[12] HemoAct may also exert beneficial effects in the treatment of microvascular perfusion disorders based on the albumin characteristics of volume expander and antioxidant.[13] We herein aim to investigate the neuroprotection of HemoAct and its underlying mechanisms in the rat tMCAO model.

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request. A detailed description of Materials and Methods can be found in the online-only Data Supplement.

Animals

All animal experiment protocols were approved by the Animal Studies Ethics Committee at the Hokkaido University Graduate School of Medicine. All procedures used in the present study were performed in accordance with the institutional guidelines for animal experimentation and the Guidelines for Proper Conduct of Animal Experiments by the Science Council of Japan. A total of 125 rats were subjected to experiments which are described in the online-only Data Supplement.

HemoAct

HemoAct is an HBOC and its physical property is described in the online-only Data Supplement.

tMCAO Model

Transient focal cerebral ischemia was induced by right MCAO using a silicone rubber-coated nylon filament. Detailed procedures are described in the online-only Data Supplement.

In Vivo Experimental Protocol

Analysis of Effects of HemoAct on tMCAO Rats After 24 Hours of Reperfusion

The effects of HemoAct on the neurological and pathological findings were investigated after 24 hours of reperfusion in the 4 (control, vehicle, 50% HemoAct, and HemoAct) groups. Detailed procedures are described in the online-only Data Supplement.

Analysis of Effects of HemoAct on tMCAO Rats During the Early Phase of Reperfusion

The effects of HemoAct on the cortical blood flow, tissue oxygen content, and microvascular perfusion were investigated during the early phase of reperfusion in the control and HemoAct groups. Detailed procedures are described in the online-only Data Supplement.

Neurological Scores

A neurological assessment was performed using an 18-point scale score. Detailed procedures are described in the online-only Data Supplement.

Evaluation of Brain Injury and Edema Volume

Infarct and edema volumes were evaluated using 2,3,5-triphenyltetrazolium chloride staining. Detailed procedures are described in the online-only Data Supplement.

Western Blotting

Western blotting was performed using anti–MMP-9 (matrix metalloproteinase-9) antibody and anti–4-hydroxynonenal (4-HNE) antibody. Detailed procedures are described in the online-only Data Supplement.

Cerebral Blood Flow Measurements

Cerebral blood flow was measured by Laser Doppler flowmetry in the middle cerebral artery territories. Detailed procedures are described in the online-only Data Supplement.

Tissue Partial Oxygen Pressure Measurement

Brain tissue partial oxygen pressure (Pt_{O_2}) was measured by an oxygen electrode method. Detailed procedures are described in the online-only Data Supplement.

Immunohistochemical Staining

Immunohistochemical staining was performed with paraffin sections of the brain fixed in 4% paraformaldehyde. Detailed procedures are described in the online-only Data Supplement.

Analysis of Microvascular Perfusion and Narrowing Changes

Microvascular perfusion and narrowing changes were examined by immunohistochemical analysis in the control and HemoAct groups. Detailed procedures are described in the online-only Data Supplement.

Analysis of the Distribution of HemoAct and Its Related Effects in the Ischemic Core

The distribution of HemoAct, IgG, and 8-hydroxy-2′-deoxyguanosine in the ischemic core was examined by immunohistochemical analysis in the control and HemoAct groups. Detailed procedures are described in the online-only Data Supplement.

In Vitro Cellular Hypoxia-Reoxygenation Injury Model

The antioxidant effects of HemoAct were examined in hypoxia/reoxygenation-treated rat brain microvascular endothelial cells (RBMECs). Detailed procedures are described in the online-only Data Supplement.

Measurement of Reactive Oxygen Species Production in RBMECs

The measurement of reactive oxygen species (ROS) production was performed using 3 different methods: dihydroethidium fluorescent staining, an 8-hydroxy-2′-deoxyguanosine ELISA, and 4-HNE Western blotting. Detailed procedures are described in the online-only Data Supplement.

Analysis of Effects of Albumin on tMCAO Rats After 24 Hours of Reperfusion

The effects of albumin itself on tMCAO rats were examined and compared with the result in the analysis of the effects of HemoAct on tMCAO rats. Detailed procedures are described in the online-only Data Supplement.

Data Collection and Statistical Analysis

All data were collected by investigators blinded to the experimental groups and were presented as means±SD. Two group comparisons were performed by the Mann-Whitney *U* test. Multiple comparisons were conducted by a 1-way ANOVA

followed by Bonferroni test or the Kruskal-Wallis test and then by the Steel-Dwass test. Sample sizes were selected based on our previous experiments. Values of P <0.05 were considered to be significant.

Results

Physiological Parameters

No significant differences were observed in the values of basic physiological parameters including blood gas and blood pressure before tMCAO or in the degree of cerebral blood flow (CBF) reductions during tMCAO between the 4 groups. Physiological parameters after the treatments were also not significantly different between the 4 groups, indicating that HemoAct did not cause adverse effects (Table II in the online-only Data Supplement).

Neurological Status and Infarct and Edema Volumes After 24 Hours of Reperfusion

The effects of HemoAct on the neurological status and infarct and edema volumes were investigated in the 4 groups (Figure 1A). No significant differences were observed in neurological scores between the 4 groups at the early phase of tMCAO or early phase of reperfusion (Figure 1B). However, the neurological status was significantly superior in the HemoAct group than in the control group (P<0.01), vehicle group (P<0.01), and 50%

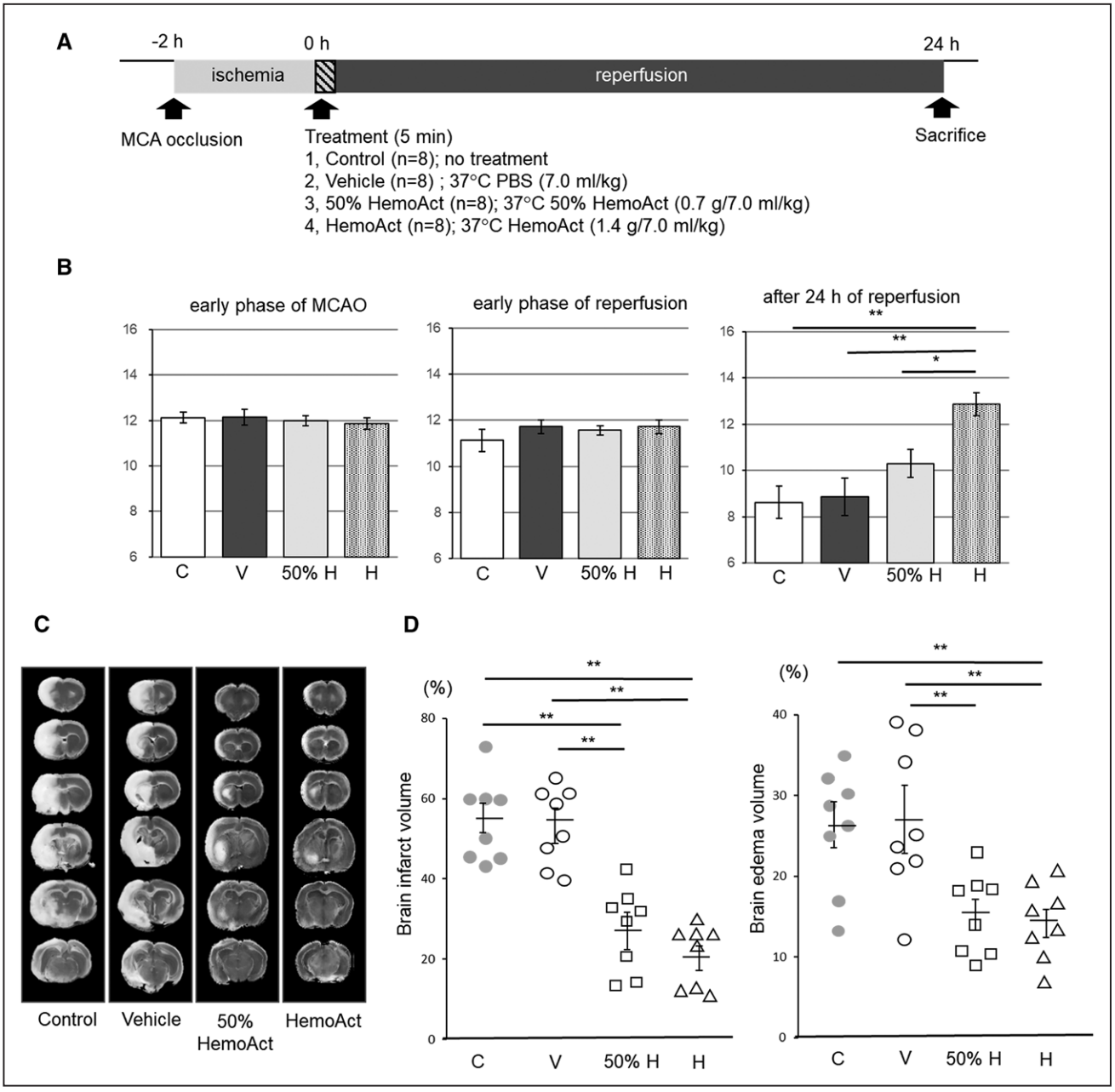

Figure 1. Effects of HemoAct on neurological findings and brain infarct and edema volumes. **A**, Experimental design diagram. **B**, Neurological function evaluated using an 18-point scale at the early phase of middle cerebral artery occlusion (MCAO), the early phase of reperfusion, and 24 hours after reperfusion. *P<0.05 and **P<0.01. **C**, Representative images of brain sections with 2,3,5-triphenyltetrazolium chloride staining. **D**, Quantitative evaluation of brain infarct and edema volumes. **P<0.01. 50% H indicates 50% HemoAct group; C, control group; H, HemoAct group; and V, Vehicle group.

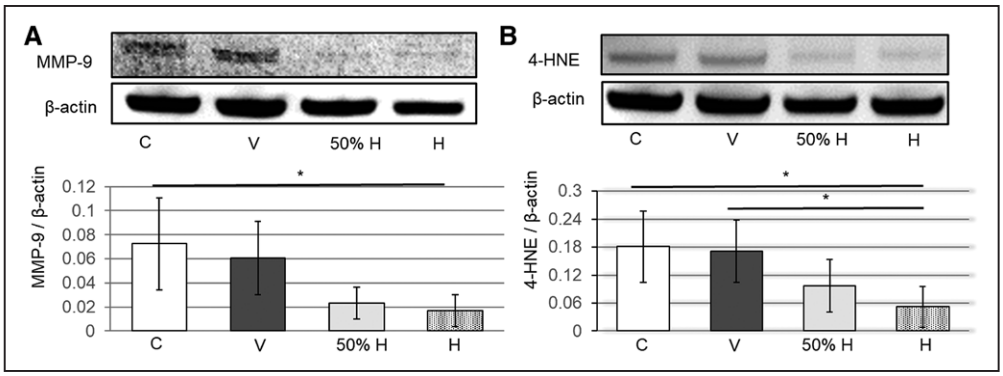

Figure 2. Effects of HemoAct on MMP-9 (matrix metalloproteinase-9) and 4-hydroxynonenal (4-HNE) production. **A**, Representative image of MMP-9 Western blotting and quantitative evaluation of immunoblots (n=5 in each group). **B**, Representative image of 4-HNE Western blotting and quantitative evaluation of immunoblots (n=5 in each group). *P<0.01. 50% H indicates 50% HemoAct group; C, control group; H, HemoAct group; and V, Vehicle group.

HemoAct group (P<0.05) after 24 hours of reperfusion (Figure 1B). Brain infarct volumes were significantly smaller in the HemoAct group (20.2±3.1%) and 50% HemoAct group (27.1±4.7%) than in the control group (55.2±3.6%; P<0.01) and vehicle group (53.2±4.3%; P<0.01; Figure 1C and 1D). The mean infarction volume of the HemoAct group was 37% that of the control group. Edema volumes were also significantly smaller in the HemoAct group than in the control group (P<0.01) and vehicle group (P<0.01; Figure 1D).

Activation of MMP-9 and Lipid Peroxidation After 24 Hours of Reperfusion

The effects of HemoAct on the activation of MMP-9 and lipid peroxidation were investigated by Western blotting of MMP-9 and 4-HNE. The immunoblot intensity of MMP-9 was significantly lower in the HemoAct group than in the control group (P<0.05; Figure 2A). The immunoblot intensity of 4-HNE was significantly lower in the HemoAct group than in the vehicle group (P<0.05) and control group (P<0.05; Figure 2B).

CBF and Tissue Pt_{o_2} in the Cortical Penumbra During the Early Phase of Reperfusion

CBF and tissue Pt_{o_2} were measured intermittently from just before tMCAO to 6 hours after reperfusion in the cortical penumbra of the control and HemoAct groups (Figure 3A). CBF and Pt_{o_2} were reduced after 6 hours of reperfusion in the control group, which indicated postischemic delayed hypoperfusion (Figure 3B and 3C). However, the reductions observed in CBF and Pt_{o_2} were suppressed in the HemoAct group, resulting in significant differences (P<0.01) in the values of CBF and Pt_{o_2} between the control and HemoAct groups after 6 hours of reperfusion (Figure 3B and 3C).

Microvascular Perfusion in the Cortical Penumbra During the Early Phase of Reperfusion

The status of microvascular perfusion in the cortical penumbra during the early phase of reperfusion was examined in the control and HemoAct groups (Figure 4A). Microvessels filled with rat autologous erythrocytes immunostained with the anti-rat Hb antibody decreased in number as time elapsed in the control group. However, microvessels filled with HemoAct

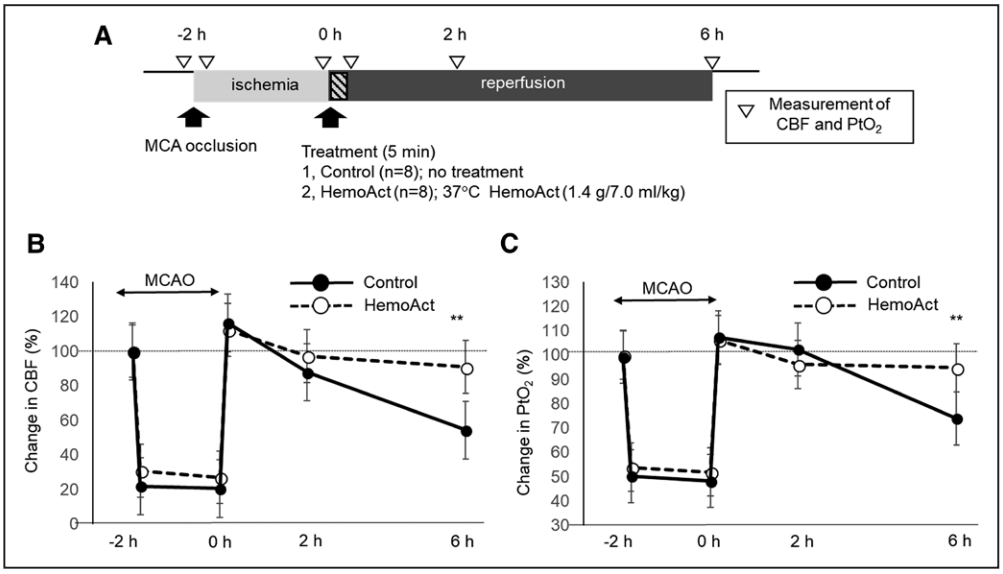

Figure 3. Effects of HemoAct on cerebral blood flow (CBF) and tissue partial oxygen pressure (Pt_{o_2}). **A**, Experimental design diagram. **B**, Time course changes in CBF in the control group and HemoAct group. **C**, Time course changes in Pt_{o_2} in the control group and HemoAct group. **P<0.01. MCAO indicates middle cerebral artery occlusion.

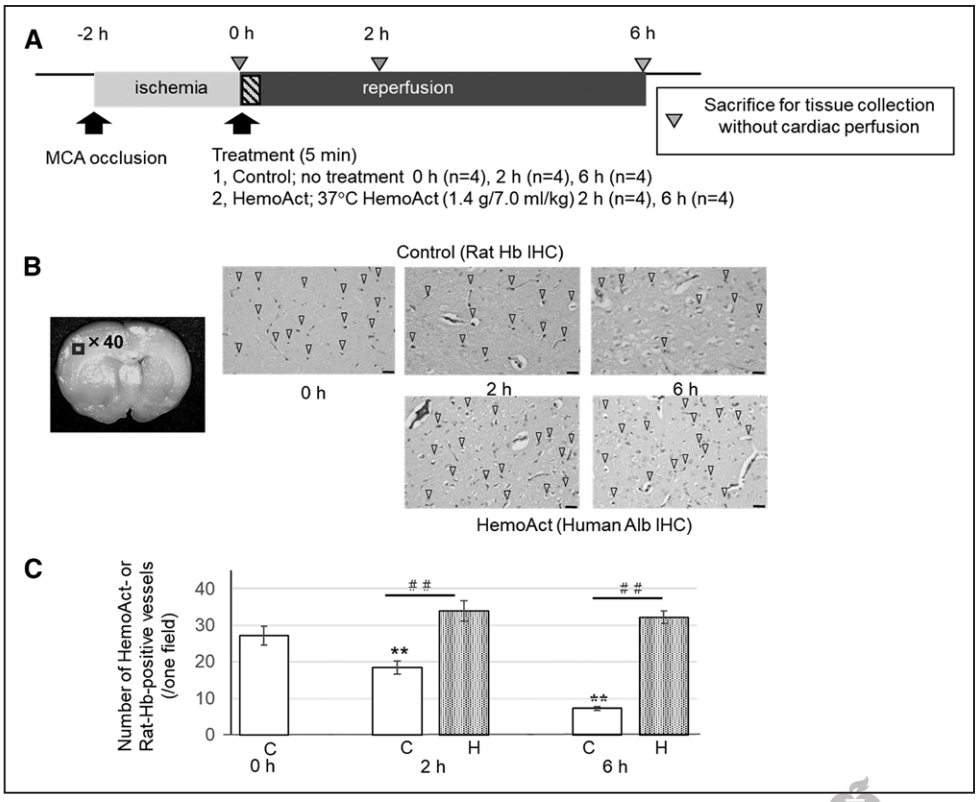

Figure 4. Effects of HemoAct on microvascular perfusion. **A**, Experimental design diagram. **B**, Representative images of immunohistochemistry with an anti-rat Hb antibody in the control group and an anti-human serum albumin (HSA) antibody in the HemoAct group. Arrowheads show rat-Hb–positive microvessels in the control group and HSA-positive (ie, HemoAct positive) microvessels in the HemoAct group. Scale bar=10 μm. **C**, Quantitative evaluation of the number of rat-Hb–positive microvessels in the control group and HSA-positive microvessels in the HemoAct group. ***P*<0.01, significantly different from the control at 0 hours. ##*P*<0.01, significant difference between 2 groups at each time point. C indicates control group; and H, HemoAct group; and MCA, middle cerebral artery.

immunostained with the anti-human albumin antibody did not decrease in number in the HemoAct group (Figure 4B). A quantitative analysis showed that the total number of Rat-Hb–positive vessels in the control group was significantly lower after 2 hours ($P<0.01$) and 6 hours ($P<0.01$) of reperfusion than at 0 hours of reperfusion. The number of positive vessels was significantly greater ($P<0.01$) in the HemoAct group than in the control group after 2 and 6 hours of reperfusion (Figure 4C).

Microvascular Narrowing Changes in the Cortical Penumbra During the Early Phase of Reperfusion

Microvascular morphological changes in the cortical penumbra during the early phase of reperfusion were examined in the control and HemoAct groups with von Willebrand factor immunohistochemistry (Figure 5A). Microvascular narrowing with an enlarged perivascular halo was observed in both groups during reperfusion (Figure 5B). A quantitative analysis showed that the cross-sectional width of microvessels was significantly smaller, whereas the cross-sectional width ratio of the perivascular halo to microvessels was significantly larger in both groups after 2 to 6 hours of reperfusion than in the control group at 0 hours of reperfusion (Figure 5C and 5D). In comparisons of the cross-sectional width of microvessels and the cross-sectional width ratio of the perivascular halo to microvessels between the control and HemoAct groups, the former was significantly greater ($P<0.01$) and the latter was significantly smaller ($P<0.01$) in the HemoAct group than in the control group after 6 hours of reperfusion (Figure 5C and 5D).

Distribution of HemoAct and Its Related Effects in the Ischemic Core During the Early Phase of Reperfusion

The distribution of HemoAct and its related effects on serum extravasation and ROS production in the ischemic core during the early phase of reperfusion was examined in an immunohistochemical analysis. HemoAct clearly extravasated into the ischemic core after 2 and 6 hours of reperfusion in most of the HemoAct-treated rats (Figure IIA in the online-only Data Supplement). HemoAct extravasation was sometimes accompanied by the extravasation of serum IgG (Figure IIA in the online-only Data Supplement). The intensity grade of IgG immunohistochemistry was somewhat, but not significantly, lower in HemoAct-treated rats than in control rats (Figure IIB in the online-only Data Supplement), suggesting that the extent of serum IgG extravasation in HemoAct-treated rats was similar or less than that in control rats. ROS production evaluated by 8-hydroxy-2′-deoxyguanosine immunohistochemistry was occasionally observed in control rats (2 out of 8 rats), but not in HemoAct-treated rats (0 out of 8 rats) (Figure IIA in the online-only Data Supplement). Therefore, HemoAct extravasation did not cause an increase in serum extravasation or ROS production in the ischemic core.

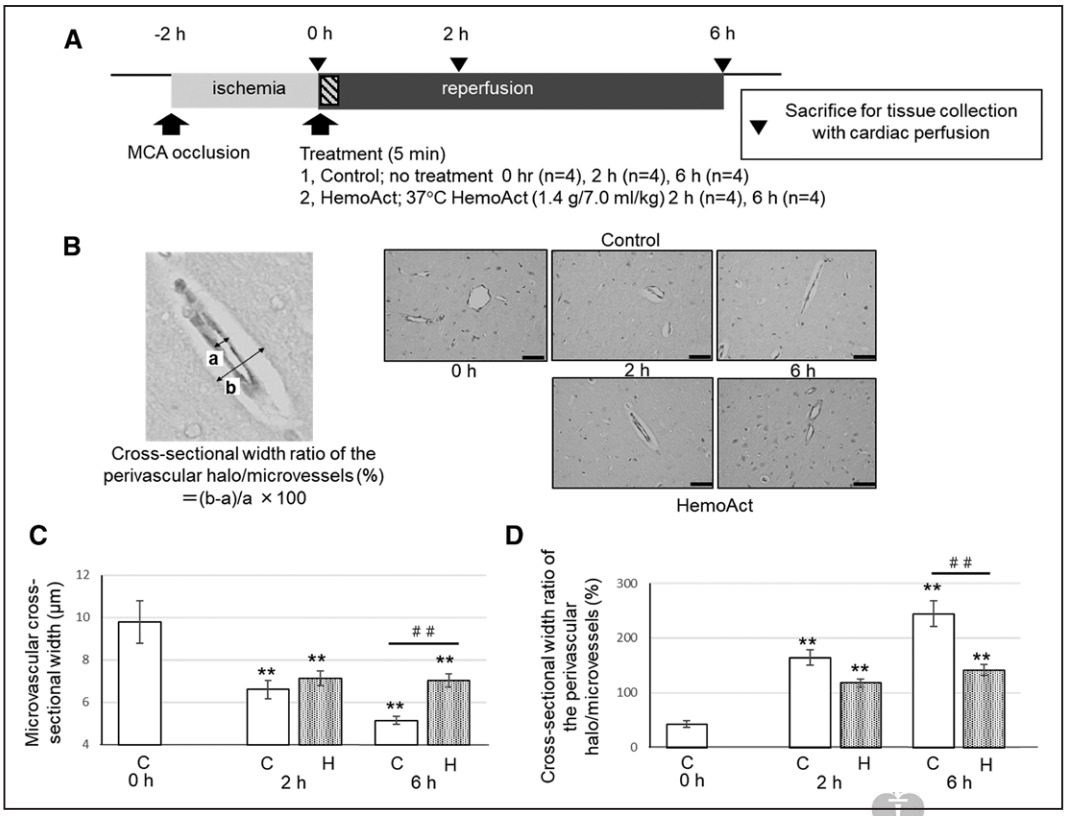

Figure 5. Effects of HemoAct on microvascular narrowing changes. **A**, Experimental design diagram. **B**, Representative images of von Willebrand factor immunoreactivity in the control group and HemoAct group. Scale bar=10 μm. **C**, Quantitative analysis of temporal changes in the cross-sectional width of microvessels. **D**, Quantitative analysis of temporal changes in the cross-sectional width ratio of microvessels to the perivascular halo. ***P*<0.01, significantly different from the control at 0 hours. ##*P*<0.01, significant difference between 2 groups at each time point. C indicates control group; H, HemoAct group; and MCA, middle cerebral artery.

Effects of HemoAct on ROS Production in Cultured RBMECs Treated With Hypoxia-Reoxygenation

The antioxidant characteristics of HemoAct were examined in cultured RBMECs treated with hypoxia-reoxygenation. We used 2 hours of 1% O_2 hypoxia and 6 hours of normoxic reoxygenation for the experimental conditions and conducted 4 treatments (control, Hb, albumin, and HemoAct) on RBMECs during the reoxygenation period (Figure 6A). Three assays to evaluate ROS production, dihydroethidium fluorescent staining, an 8-hydroxy-2′-deoxyguanosine ELISA, and 4-HNE Western blotting, showed similar results in this experiment. Although ROS production in the Hb group was similar to that in the control group, it was significantly lower in the albumin and HemoAct groups than in the control group, suggesting that HemoAct has similar antioxidant characteristics to albumin (Figure 6B through 6D).

Discussion

HBOCs were originally studied and developed as a substitute for blood transfusion for blood loss because of injury and surgery. However, because HBOCs were found to have better and faster oxygenation capabilities than erythrocytes,[14,15] the therapeutic potential of HBOCs in ischemic diseases has been actively examined.[6–8,16] These studies demonstrated that HBOCs were beneficial for providing sufficient microcirculation and ameliorating I/R injury. We also previously reported that the infusion of liposome-encapsulated Hb exerted neuroprotective effects with superior microvascular perfusion in a rat tMCAO model.[9,10] In line with these findings, we showed that HemoAct ameliorated neurological disorders and brain infarction because of I/R injury in the present study. Therefore, HBOCs have therapeutic potential for ischemic diseases with microvascular perfusion disorders.

Postischemic microvascular perfusion disorders after delayed recanalization are complex and sometimes exhibit contradictory responses, namely hyperperfusion or hypoperfusion.[17–19] Hyperperfusion is considered to increases in permeability and cellular extravasation, leading to the formation of edema and hemorrhagic transformation.[20,21] On the other hand, hypoperfusion sometimes occurs after hyperperfusion, resulting in secondary ischemia because of a microvascular perfusion disturbance. This phenomenon has been known as the no-reflow phenomenon, which was demonstrated as microvascular occlusion by leukocyte adhesion, platelet accumulation, and fibrin formation in a primate transient ischemic model.[22] However, another potential cause of microvascular perfusion disturbances was recently demonstrated in electron microscopic studies. Microvessels were transiently compressed and narrowed by swollen astrocyte end-feet after several hours of reperfusion, which may reduce microvascular perfusion.[23,24] In the present study, we observed delayed postischemic hypoperfusion, represented as reductions in cortical CBF and Pt_{O_2} and a decrease in the appearance of erythrocytes in microvessels, which showed narrowing changes with

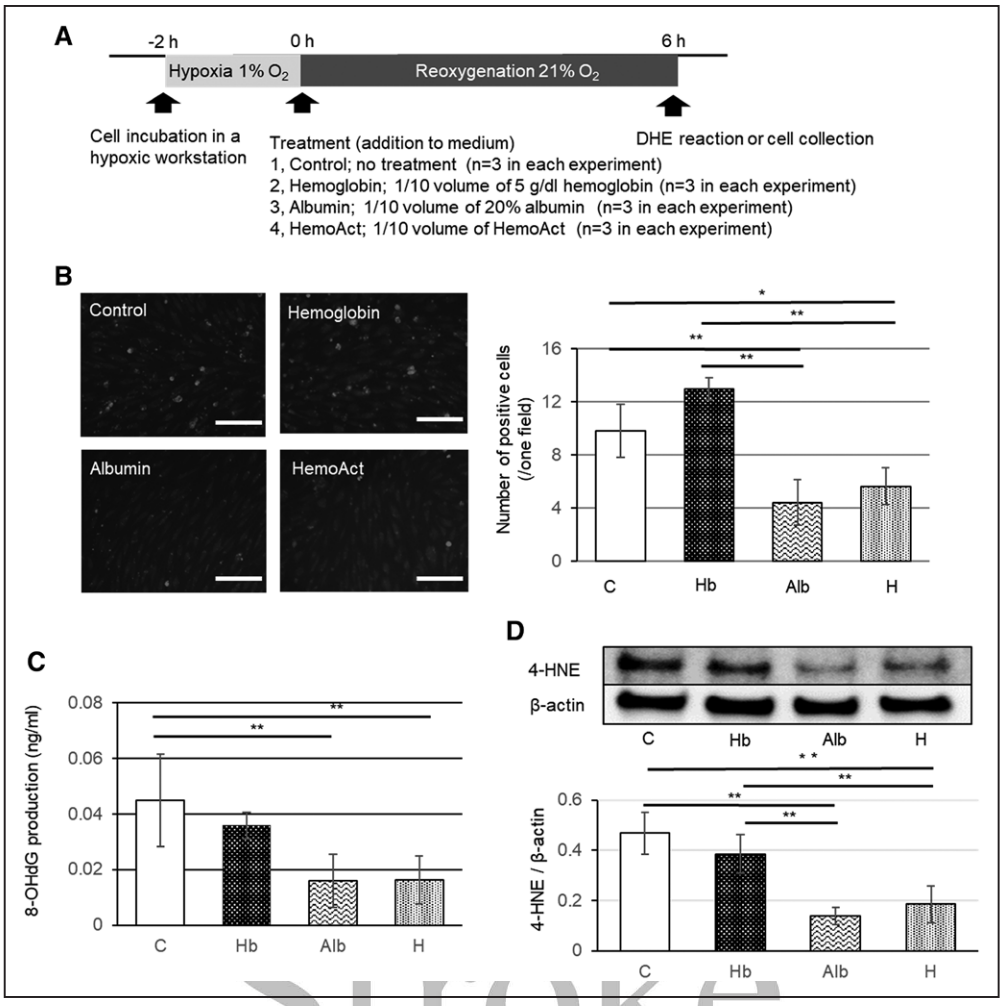

Figure 6. Effects of HemoAct on reactive oxygen species (ROS) production in cultured rat brain microvascular endothelial cells (RBMECs) treated with hypoxia-reoxygenation. **A**, Experimental design diagram. **B**, Representative fluorescent images of ROS production in RBMECs evaluated by dihydroethidium (DHE) staining (×400, Scale bar=50 μm) and quantitative evaluation of fluorescent intensity. **C**, Quantitative evaluation of 8-hydroxy-2′-deoxyguanosine (8-OHdG) production. **D**, Representative image of 4-hydroxynonenal (4-HNE) Western blotting and quantitative evaluation of immunoblots. *P<0.05 and **P<0.01. Alb indicates albumin group; C, control group; H, HemoAct group; and Hb, hemoglobin group.

an enlarged perivascular space after 6 hours of reperfusion. Considering the characteristics of HBOCs with small particle sizes, the conditions observed with microvascular narrowing changes in the I/R region are suitable for treatments with HBOCs to rescue brain tissue from irreversible damage.

To date, many HBOCs under development[25–28] have not yet been approved for clinical use because they cannot avoid increasing blood pressure because of the capture of nitric oxide by Hb. On the other hand, HemoAct covered with albumin shells has shown no increase of blood pressure in normal animals[12] and even in the I/R injury model in the present study. On the basis of the characteristics of albumin, such as a negative net charge and high electrostatic repulsion, HemoAct may prevent to capture nitric oxide and increase blood pressure. In addition, albumin exerts neuroprotective effects on cerebral I/R injury because of its functions as a volume expander and antioxidant.[29,30] As shown in Figure III in the online-only Data Supplement, 20% albumin, which was a comparable total protein mass concentration of albumin (20%=20 g/dL) to that in the administrated HemoAct (20 g/dL), alone moderately reduced infarction volumes in the same I/R injury model. Therefore, it is reasonable to consider HemoAct to have additive neuroprotective effects over other HBOCs. In the present study, we demonstrated the suppression of ROS production in in vivo and in vitro experiments (Figures 2B and 6). Therefore, HemoAct has an advantage for the treatment of ischemic diseases among HBOCs.

Albumin, which is either endogenous or exogenous, is extravasated into parenchyma and is taken up by neurons in rodent transient ischemic models.[31,32] Although it has been unclear whether the extravasation and neuronal uptake of albumin are directly related to its neuroprotective effects, the administration of exogenous albumin exerted apparent neuroprotection without a change in the degree of IgG extravasation.[31] This finding is similar to the present result showing that HemoAct extravasation did not cause an increase in IgG extravasation in the ischemic core, suggesting that HemoAct extravasation is a phenomenon based on albumin characteristics and does not enhance the deterioration of microvascular integrity and parenchymal cell viability. Although there are concerns that extracellular Hb results in an oxidative stress condition and neurotoxicity,[33] HemoAct extravasation

observed in the present study was not associated with ROS production, at least during the examination period (Figure II in the online-only Data Supplement; Figure 2B). Therefore, HemoAct extravasation in the ischemic core does not seem to have any adverse effects.

It is important to clarify how to apply HemoAct in stroke clinical practice. The experimental model we used in the present study is similar to patients who are treated with endovascular thrombectomy and receive transarterial drug infusions through a catheter after recanalization of the occluded artery. The reason why we selected a transarterial drug infusion was because we aimed to deliver HemoAct to postischemic tissue as rapidly and at as high a volume as possible. On the basis of the results of the present study, the transarterial HemoAct infusion had no adverse effects.

The limitations of the present study are as follows. The follow-up period was limited to the acute phase, up to 24 hours of reperfusion. One reason for the time limitation was the high death rate in this model after >24 hours of reperfusion. In future preclinical studies, long-term follow-ups will be needed using a less severe ischemic model. The next limitation is a lack of information on glucose levels, which is related to reperfusion state in stroke models (vascular effects of hyperglycemia) and could be 1 reason for the poor reperfusion. Another limitation is that experiments with different ischemic models, such as a permanent ischemic model, and different administration routes, including an intravenous infusion, need to be performed to expand indications for the HemoAct treatment. Furthermore, the long-term safety of HemoAct not only in normal animals but also in animals with cerebral ischemia is another issue that needs to be clarified.

In conclusion, HemoAct exerted strong neuroprotective effects on short-term I/R injury without Hb adverse effects. Superior microvascular perfusion and O_2 transport as well as possible antioxidant effects seem to be the underlying neuroprotective mechanisms against I/R injury. HemoAct has potential as an HBOC in the treatment of ischemic diseases.

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Disclosures

None.

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Novel Hemoglobin-Based Oxygen Carrier Bound With Albumin Shows Neuroprotection With Possible Antioxidant Effects

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SUPPLEMENTAL MATERIAL

Materials and Methods

All animal experiment protocols were approved by the Animal Studies Ethics Committee at the Hokkaido University Graduate School of Medicine. All procedures used in the present study were performed in accordance with the institutional guidelines for animal experimentation.

Animals

A total of 125 wild-type male Sprague-Dawley rats (CLEA Japan, Inc., Tokyo, Japan) weighing 260 to 310 g were used in experiments. Male rats were employed to eliminate estrogen-mediated neuroprotective effects on ischemic injury. Animals were housed in a controlled environment (25°C, 50% humidity, and 12-hour light-dark cycle) and allowed free access to food and water. Nine animals were excluded due to insufficient reductions in cerebral blood flow (CBF) during transient middle cerebral artery occlusion (tMCAO) (n=5), filament-induced subarachnoid hemorrhage (n=3), and lethal intraoperative complications (n=1). There was no mortality during the 24-hour observation period in any treatment groups.

HemoAct™

HemoAct is an artificial O_2 carrier that is composed of a core-shell protein cluster comprising bovine hemoglobin (Hb) in the center and human serum albumin (HSA) at the periphery (**Supplemental figure IA)** [1]. The amino groups of the surface lysines on Hb were covalently connected with a thiol group of unique Cys-34 on HSA by an α-succinimidyl-ε-maleimide cross-linker (average HSA/Hb ratio = 3.0 ± 0.2). HemoAct solution ([Hb] = 5 g/dL ([total protein] = 20 g/dL) in phosphate-buffered saline [PBS], pH 7.4) was prepared according to our previously reported procedure [2]. Since the core Hb was wrapped sufficiently by three HSAs, HemoAct had a low isoelectric point (pI) of 5.1. The O_2 affinity of HemoAct (P50 = 9 Torr) was higher than that of native Hb (P50 = 23 Torr) (**Supplemental figure IB**). We previously demonstrated the preclinical safety of this formulation as a red blood cell substitute in animal studies [3].

Transient MCAO model

Rats were anesthetized with isoflurane at initial and maintenance concentrations of 4.0% and 1.0-1.5% respectively, in 70% N_2O and 30% O_2 gas through a facial mask. Transient focal cerebral ischemia was induced by right MCAO using a silicone rubber-coated nylon filament with a tip diameter of 0.37 mm (Doccol Corp., Redlands, CA, USA). Rectal temperature was monitored and maintained at 37±03°C using a heating pad during the operative procedure. The right carotid artery (CA) was surgically exposed, and the external CA was then ligated, cut, and reversed proximally. The filament was inserted into the external CA and advanced into the internal CA in order to block the origin of the MCA. CBF measurements and a neurological scoring assessment were performed under awake conditions to verify successful MCAO. CBF in the territory of MCA was measured before and after MCAO. Rats with a CBF reduction of 70% and Bederson score of 3 points or higher were included in subsequent experiments. After 2-hour MCAO, the filament was gently withdrawn to provide reperfusion. When collecting brain specimens at the end of experiments, rats were deeply anesthetized to prevent pain and discomfort.

In vivo experimental protocol

a) Analysis of effects of HemoAct on tMCAO rats after 24 hours of reperfusion

Rats were subjected to 2-hour MCAO and then divided into the following groups (n=13 in each group); (1) a HemoAct group infused with 37°C HemoAct (1.4 g/7 ml/kg) through a micro-catheter placed in the right ICA immediately prior to reperfusion for 5 minutes, (2) a 50% HemoAct group infused with 37°C HemoAct diluted 2-fold (0.7 g/7 ml/kg) in the same manner as the HemoAct group, (3) a vehicle group infused with 37°C solvent PBS (7 ml/kg) in the same manner as the HemoAct group, and (4) a control group subjected to recanalization only without infusion. Physiological parameters, neurological scores, infarct and edema volumes, and the production of protease and reactive oxygen species (ROS) after 24 hours of reperfusion were then evaluated in these 4 groups. A blood gas analysis (pCO_2, pO_2, pH, Hb, and Ht) and blood pressure were monitored twice at the beginning of MCAO and the end of reperfusion. The concealment of treatment was not possible during the operation due to color differences in the reagents (HemoAct; dark red and vehicle; colorless). However, subsequent analyses were performed with blinded methods.

b) Analysis of effects of HemoAct on tMCAO rats during the early phase of reperfusion

Since ischemia-reperfusion induced microvascular narrowing changes due to astrocyte end-foot swelling with several hours of reperfusion [4, 5], which may be related to post-ischemic delayed hypoperfusion, we attempted to examine changes in microvascular perfusion during the early phase of reperfusion (after 2 and 6 hours of reperfusion), and the effects of HemoAct on these changes. In order to achieve this, we employed 2-hour MCAO rats and divided them into two groups: the HemoAct and control groups. CBF and tissue partial oxygen pressure (PtO_2) were measured intermittently under anesthetic conditions from the onset of MCAO to 6 hours after reperfusion in the cortical penumbra (described in detail in the following section). We then analyzed the microvascular perfusion of HemoAct in the HemoAct group and autologous erythrocytes in the control group with an immunohistochemical technique (described in detail in the following section). Rats (n=20) were not subjected to cardiac perfusion before brain tissue collection (Fig. 4a). We also analyzed microvascular narrowing changes by measuring cross-sectional widths and the cross-sectional width ratio of the perivascular halo/microvessels between the HemoAct and control groups with an immunohistochemical technique (described in detail in the following section). Rats (n=20) were subjected to cardiac perfusion with 4% paraformaldehyde in PBS before brain tissue collection (Fig. 5a). In addition, we analyzed the distribution of HemoAct and its related effects in the ischemic core by comparing between the HemoAct and control groups with an immunohistochemical technique (described in detail in the following section). We used sections from the same rats (n=40), which were used in the analysis of microvascular perfusion and microvascular narrowing changes as described above.

Neurological scores

A neurological assessment (n=8 in each group) was performed under awake conditions after the onset of tMCAO, after the onset of reperfusion, and 24 hours after the onset of reperfusion using an 18-point scale score by an observer blinded to the grouping information of rats. The score was graded on a scale of 0 to 18; rats with low scores had severe neurological impairments [6].

Evaluation of brain injury and edema volume

Infarct and edema volumes were evaluated using 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) staining. Brains (n=8 in each group) were sectioned coronally, and six serial 2-mm-thick sections were obtained and immersed in 2%

TTC at 37°C for 20 minutes. Brain sections were photographed and quantitatively analyzed with ImageJ software (Image J 1.37v; NIH, Bethesda, MD, USA) by an observer blinded to the grouping information.

Brain infarct (%) = (contralateral hemispheric volume – ipsilateral non-infarct volume)/contralateral hemispheric volume

Edema volume (%) = (ipsilateral hemispheric volume – contralateral hemispheric volume)/contralateral hemispheric volume

Western blotting

Western blotting was performed using an anti-matrix metalloproteinase-9 (MMP-9) antibody (Cell Signaling Technology, Danvers, MA, USA) and anti-4-hydroxynonenal (4-HNE) antibody (Abcam, Cambridge, MA, USA) to evaluate the expression of protease and production of ROS. Proteins were extracted from brain tissue (n=5 in each group) of the ipsilateral hemisphere by homogenizing in RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA). An equal amount of total protein (10 μg) was electrophoresed in a NuPage 4-12% Bis-Tris Gel (Life Technologies, Carlsbad, CA, USA). The membrane was blocked with 10% non-fat dry milk in PBS containing 0.05% Tween-20 at room temperature for 1 hour, followed by an incubation with the primary antibody at 4°C overnight (anti-MMP-9 rabbit polyclonal antibody; 1:2000, anti-4-HNE rabbit polyclonal antibody; 1:2000). After washing with PBS containing 0.05% Tween-20, the membrane was incubated with a peroxidase-conjugated secondary antibody at room temperature for 1 hour. Labeled proteins were detected using chemical luminescence (ECL Advanced Western Blotting Detection Kit; GE Healthcare Life Science). Immunoblots were quantified by densitometry using ImageJ software.

CBF measurements

Laser Doppler flowmetry (OMEGA FLOW FLO-C1; OMEGAWAVE, Tokyo, Japan) was used to measure cortical microvascular CBF in the MCA territories. The probe was placed over the skull 5 mm lateral to the midline and 2 mm posterior to the bregma in order to measure CBF changes during tMCAO and reperfusion.

PtO_2 measurement

An oxygen electrode (POG-203, Unique Medical, Tokyo, Japan) was used to measure brain tissue PtO_2. The oxygen electrode was calibrated at 150 mmHg in water aerated with air. A burr hole was made 3 mm lateral to the midline and 2 mm posterior to the bregma. The electrode was inserted to a depth of 2 mm from the brain surface to measure PtO_2 during MCAO and reperfusion.

Immunohistochemical staining

Paraffin sections of the brain fixed in 4% paraformaldehyde were used for immunohistochemistry. Four-micrometer-thick coronal sections located between 1 mm anterior and 1 mm posterior to the bregma were prepared. The antibodies used for immunohistochemical staining were an anti-rat Hb polyclonal antibody (1:40 at room temperature for 1 hour, Cloud-Clone Corp., TX, USA), anti-human albumin antibody (1:200 at room temperature for 1 hour, Abnova, Taipei, Taiwan), anti-von Willebrand Factor (vWF) antibody (1:200 at room temperature for 1 hour, Chemicon International Inc., Billerica, MA, USA), and anti-rat immunoglobulin G (IgG) antibody (1:1000 at room temperature for 1 hour, GeneTex, CA, USA). After the first antibody incubation under the conditions described above, sections were treated with enzyme-conjugated polymer reagent (Dako EnVision+Kit, DakoCytomation, Glostrup, Denmark) for 1 hour. The 3,3'-Diaminobenzidine (DAB) chromogen was applied for 2 to 3 minutes in order to obtain a chromogenic signal. In 8-hydroxy-2'-deoxyguanosine (8-OHdG) immunohistochemical staining, the avidin-biotin-alkaline phosphatase complex (ABC-AP) technique was used in accordance with the manufacturer's instructions. After an incubation with 0.1 μg/mL of an anti-8-OHdG monoclonal antibody (clone N45.1, Nikken SEIL Co., Japan) at 4°C overnight, sections were treated with biotin-labeled rabbit anti-mouse IgG serum (1:300 at room temperature for 30 minutes, DakoCytomation, Glostrup, Denmark). After an incubation with the ABC-AP complex (1:100 at room temperature for 30 minutes, Vector Lab., Burlingame, CA), the substrate for AP was applied for 20 to 30 minutes in order to obtain a chromogenic signal.

Analysis of microvascular perfusion

The status of microvascular perfusion in the early phase of reperfusion (after 2 and 6 hours of reperfusion) was compared between the control and HemoAct groups. We counted microvessels filled with rat autologous erythrocytes that were immunostained with the anti-rat

Hb antibody in the control group (after 0, 2, and 6 hours of reperfusion; n=4 in each time-point group) and microvessels filled with HemoAct immunostained with the anti-human albumin antibody in the HemoAct group (after 2 and 6 hours of reperfusion; n=4 in each time-point group) using ImageJ software at 4 randomly selected 400× magnified fields in the cortical penumbra. The average number of microvessels per field was used for the quantitative analysis.

Analysis of microvascular narrowing changes

Microvascular narrowing changes were examined by vWF immunohistochemistry as described previously [4] and were compared between the control and HemoAct groups. The cross-sectional width (the minimum transverse width) of a microvessel and its adjacent lucent perivascular halo were measured with the aid of software measuring function at 4 randomly selected 400× magnified fields in the cortical penumbra. All microvessels with a cross-sectional width ≤15 μm were selected in the fields for use in the quantitative analysis. The cross-sectional width ratio of the perivascular halo/microvessels was calculated as (width of the perivascular halo – width of the microvessel)/ width of the microvessel × 100 (%). The temporal profile of microvascular narrowing changes was evaluated in the control group (after 0, 2, and 6 hours of reperfusion; n=4 in each time-point group) and HemoAct group (after 2 and 6 hours of reperfusion; n=4 in each time-point group).

Analysis of the distribution of HemoAct and its related effects in the ischemic core

The distribution of HemoAct, IgG, and 8-OHdG in the ischemic core was examined by immunohistochemical staining. The temporal profile of distribution was evaluated in the control group (after 0, 2, and 6 hours of reperfusion; n=8 in each time-point group) and HemoAct group (after 2 and 6 hours of reperfusion; n=8 in each time-point group). The intensity of immunostaining was graded as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong) by an observer blinded to the grouping information of rats.

In vitro cellular hypoxia-reoxygenation injury model

Rat brain microvascular endothelial cells (RBMECs) purchased from Cell Applications, Inc. were cultured according to the manufacturer's instructions. RBMECs at passages 4 to 6 were used in the hypoxia-reoxygenation experiment. Hypoxic conditions (1% O_2, 5% CO_2, and 94%

N_2 at 37°C) were generated by a hypoxia workstation (InvivoO_2 300, Baker Ruskinn, Maine, USA). RBMECs were incubated under the hypoxic conditions in the workstation for 2 hours. In order to produce hypoxia rapidly, at the start of the hypoxia cell incubation, we changed the original medium to the prepared hypoxic medium, which was incubated overnight under hypoxic conditions. Reoxygenation was achieved by returning RBMECs to the normoxic incubator and changing to the normoxic medium containing the respective reagents as described in the following protocol.

In vitro experimental protocol

After 2 hours of the hypoxia treatment, RBMECs were divided into the following groups (n=3 in each group): (1) a HemoAct group incubated with medium containing a 1/10 volume of HemoAct, (2) an albumin group incubated with medium containing a 1/10 volume of 20% albumin solution, (3) a Hb group incubated with medium containing a 1/10 volume of 5 g/dl Hb solution, and (4) a control group incubated with medium containing no reagent. After a 6-hour incubation of each treatment under normoxic conditions at 37°C, the treated RBMECs were subjected to the following procedures to measure ROS production (Fig. 6A).

Measurement of ROS production in RBMECs

The measurement of ROS production was performed using 3 different methods: dihydroethidium (DHE) fluorescent staining, an 8-OHdG enzyme-linked immunosorbent assay (ELISA), and 4-HNE Western blotting. DHE fluorescent staining was performed by incubating RBMECs with 5 μM DHE (Invitrogen, Oregon) at 37°C for 30 min. After fixation by 4% paraformaldehyde for 10 minutes, red fluorescence in cells was detected in 25 randomly selected fields (×100) by a fluorescence microscope (BZ-X710; Keyence, Osaka, Japan). The intensity of red fluorescence was quantified using ImageJ software. 8-OHdG ELISA was performed with a highly sensitive 8-OHdG assay kit (Nikken SEIL Co., Japan) following the manufacturer's instructions. DNA extraction from RBMECs and the measurement of 8-OHdG levels in DNA extracts were performed as described in the manual provided by the supplier. 4-HNE Western blotting was performed using an anti-4-HNE antibody (Abcam, Cambridge, MA, USA) with protein extracts (5 μg) from RBMECs. The method of Western blotting was the same as that described above.

Analysis of effects of albumin on tMCAO rats after 24 hours of reperfusion

In order to examine the effects of albumin on tMCAO rats, rats (n=8) were subjected to 2-hour MCAO and were infused with 37°C 20% albumin (7 ml/kg) through a micro-catheter placed in the right ICA immediately prior to reperfusion for 5 minutes. The molar amount of albumin in infused 20% albumin was similar to that of albumin in infused HemoAct in the analysis of the effects of HemoAct on tMCAO rats. Infarct volumes after 24 hours of reperfusion in albumin-treated rats were evaluated and then compared with those of the HemoAct and control groups in the analysis of the effects of HemoAct on tMCAO rats.

Data collection and statistical analysis

All data were collected by investigators blinded to the experimental groups and were presented as means ± SD. Two group comparisons were performed by the Mann-Whitney U test. Multiple comparisons were conducted by a one-way analysis of variance followed by Bonferroni's test or the Kruskal-Wallis test and then by the Steel-Dwass test. Sample sizes were selected based on our previous experiments. Values of $p<0.05$ were considered to be significant.

Supplemental table I

Stroke Online Supplement

Table I. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

Methodological and Reporting Aspects	Description of Procedures
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. ☑ 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.
Experimental details, ethics, and funding statements	☑ 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.

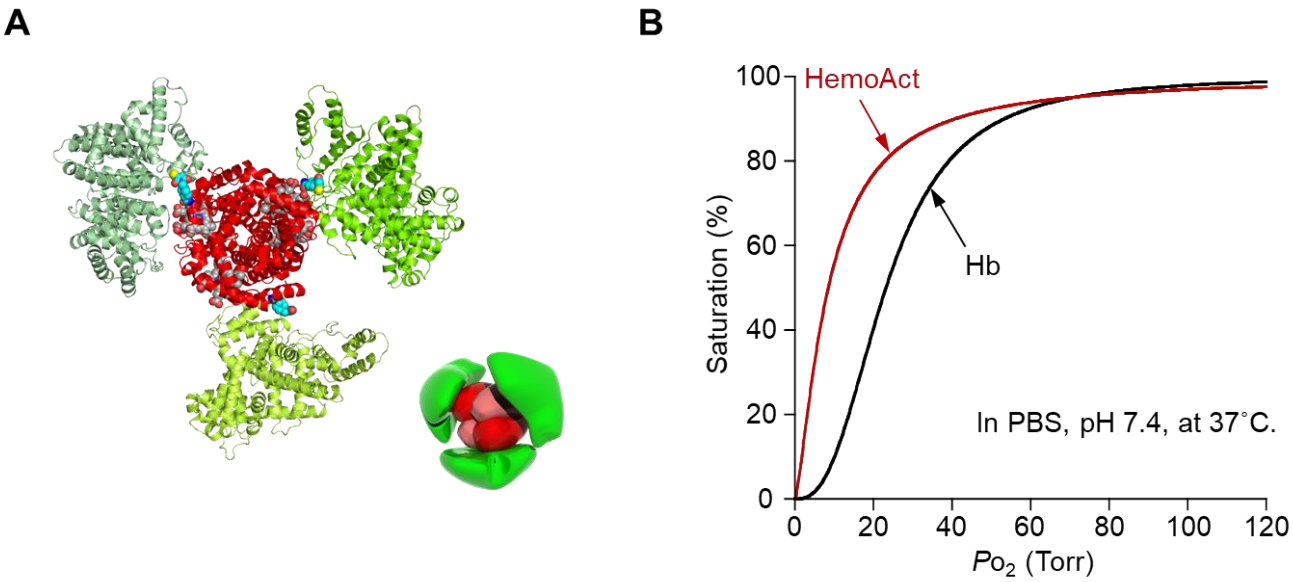
Supplemental table II

	Control n=8	Vehicle n=8	50%HemoAct n=8	HemoAct n=8	
Before MCAO					
Mean ABP (mmHg)	89.3±20.1	94.9±20.8	94.5±11.5	88.9±18.3	n.s.
pH	7.49±0.07	7.51±0.03	7.49±0.04	7.46±0.04	n.s.
pCO2 (mmHg)	28.6±3.3	29.4±4.3	31.0±5.9	33.3±4.2	n.s.
pO2 (mmHg)	159.1±10.3	157.1±8.4	145.6±17.7	144.0±24.0	n.s.
Ht (%)	39.1±7.1	35.3±2.2	36.6±3.8	39.4±2.6	n.s.
Hb (mg/dl)	12.7±2.4	11.4±0.7	11.9±1.3	12.8±0.9	n.s.
Reduction in CBF from the previous value(%)	19.2±6.5	20.4±4.0	21.5±7.0	23.0±5.7	n.s.
After 24hrs of reperfusion					
Mean ABP (mmHg)	92.9±19.0	98.7±23.2	95.9±31.2	93.4±16.4	n.s.
pH	7.35±0.1	7.46±0.04	7.45±0.03	7.43±0.1	n.s.
pCO2 (mmHg)	48.9±10.7	37.9±10.2	41.1±7.6	39.4±12.3	n.s.
pO2 (mmHg)	193.1±66.9	175.7±7.2	168.1±11.0	158.6±29.1	n.s.
Ht (%)	41.0±3.7	37.1±2.3	40.0±5.4	39.1±2.3	n.s.
Hb (mg/dl)	12.3±1.1	12.4±1.2	11.7±0.9	12.1±0.9	n.s.

All values are the mean±SD. n.s means not significant.

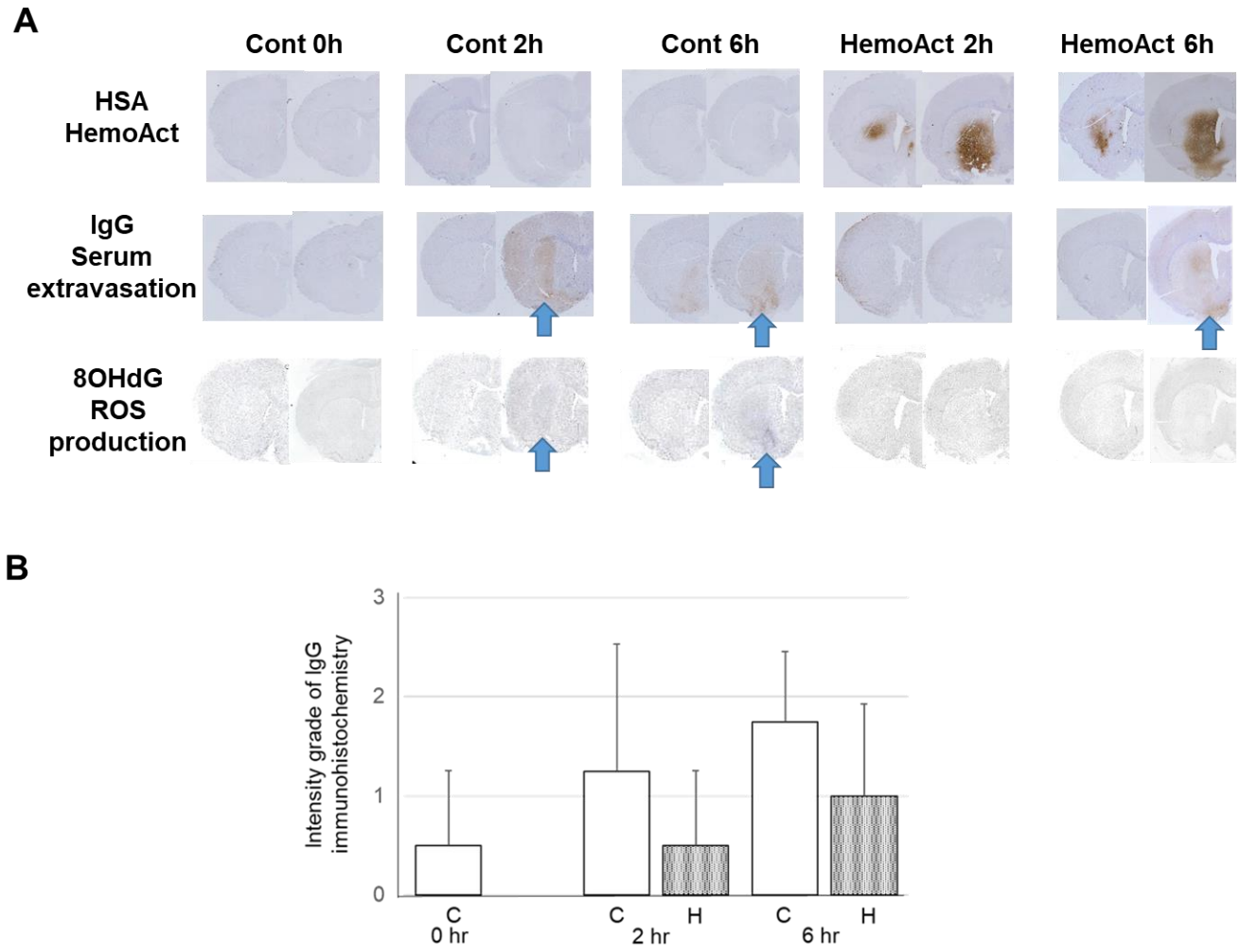
Supplemental table. Physiological parameters and percentages of cerebral blood flow reductions in four groups.

Supplemental figure I



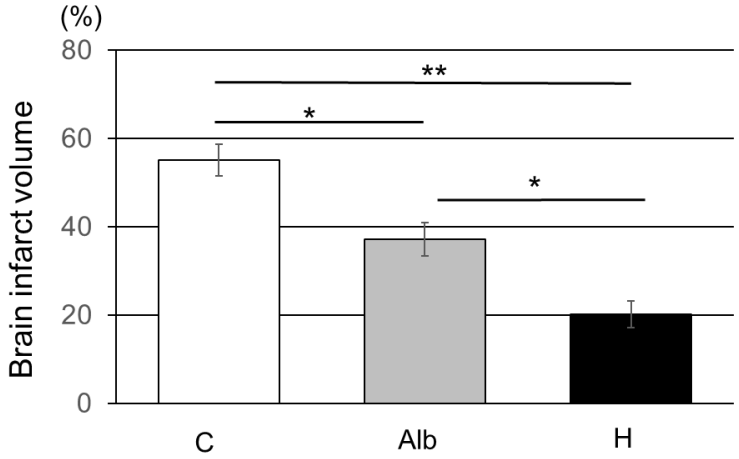
Supplemental figure I. Material characteristics of HemoAct. A, Molecular structure of HemoAct. A hemoglobin (shown in red) core is wrapped by 3 human serum albumins (shown in green) with the formation of covalent bonds (shown in blue). **B**, Oxygen equilibrium curve of HemoAct and hemoglobin (Hb)

Supplemental figure II



Supplemental figure II. Distribution of HemoAct, immunoglobulin G (IgG), and 8-hydroxy-2'-deoxyguanosine (8-OHdG) in the ischemic core during the early phase of reperfusion. A, Representative images (2 individuals in each time-point) of immunohistochemistry with an anti-human serum albumin antibody, anti-IgG antibody, and anti-8OHdG antibody in the control and HemoAct groups **B**, Quantitative evaluation of the intensity grade of IgG immunohistochemistry. n=8 in each time-point group. C indicates the control group; and H indicates the HemoAct group.

Supplemental figure III



Supplemental figure III. Effects of albumin on brain infarct after 24 hours of reperfusion. Quantitative evaluation of brain infarct volumes (n=8 in each group). *P<0.05 and **P<0.01. C indicates the control group; Alb indicates the albumin group; and H indicates the HemoAct group.

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