Liposome-Encapsulated Hemoglobin Reduces the Size of Cerebral Infarction in the Rat
Evaluation With Photochemically Induced Thrombosis of the Middle Cerebral Artery

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Background and Purpose—Liposome-encapsulated hemoglobin (LEH; TRM-645) is a novel oxygen (O2) carrier with a lower O2 affinity (P50O2/H11005 40 mm Hg) than red blood cells. In contrast to cell-free hemoglobin, encapsulation prevents hemoglobin extravasation, whereas its subcellular size (230 nm) may improve O2 delivery and limit the severity of cerebral infarction.

Methods—The extent of cerebral infarction was determined 24 hours after photochemically induced thrombosis of the middle cerebral artery from the integrated area of infarction detected by triphenyltetrazolium chloride staining in rats receiving no treatment, 10 mL/kg of LEH, homologous blood, empty liposomes, or saline. To develop a dose-response relationship, LEH dose was reduced from 10 mL/kg to 2 mL/kg, 0.4 mL/kg, and 0.08 mL/kg.

Results—Infarction extent was significantly suppressed in rats receiving LEH as compared with animals receiving no infusion, saline, empty liposome, or transfusion in the cortex but not in the basal ganglia, where all had similar degrees of damage. The dose-response relationship revealed that as little as 2 mL/kg of LEH was protective, whereas the total blood O2 content, hemoglobin level, and transfusion and/or infusion of empty liposomes or saline were not effective.

Conclusions—Our results suggest that LEH significantly reduces the area of infarction in the cortex but not in basal ganglia after photochemically induced thrombosis of the middle cerebral artery in the rat. (Stroke. 2007;38:1626-1632.)

Key Words: cerebral infarction □ microcirculation □ oxygen delivery □ reperfusion

Impairment of microcirculation after acute focal ischemia is a major factor in the pathogenesis of cerebral infarction.1 Oxygen (O2) delivery to areas of impending infarction is important in limiting the extent and severity of infarction, reducing neurological sequelae, and increasing patient survival. O2 supply to such ischemic penumbrae is mainly supported by microcirculation through the capillaries, which is believed to be insufficient because of underdevelopment and a delay in recruitment.2 Recent studies3,4 have demonstrated that plasma can perfuse and supply necessary metabolites even to the core of ischemia, but delivery of soluble O2 is limited unless hyperbaric O2 therapy is used.5 Although perfluorocarbon has been tried in acute cerebral ischemia,6,7 its efficacy remains inconsistent. Recently, cell-free hemoglobin8–10 has been developed as an artificial O2 carrier; renal clearance and hypertensive response obscured neuroprotective effects in experiments8,9 and in a human trial.10 Liposome-encapsulated hemoglobin (LEH)11,12 may be effective in acute brain ischemia, because its size (230 nm) may not only prevent extravasation but also allow O2 delivery beyond the obstruction with plasma to areas where red blood cells (RBCs) seldom reach, thereby reducing the O2 diffusion distance. Sigmoid O2 dissociation characteristics may allow more efficient O2 delivery in room air than perfluorocarbon, which needs a higher O2 inspiration because of its linear O2 dissociation capacity.6,7 Structural similarity to RBC allows coencapsulation of an allosteric effector to modulate O2 affinity.11 The objective of this study is to evaluate the effects of LEH on photochemically induced thrombosis13,14 of the middle cerebral artery (MCA) in the rat.

Materials and Methods

LEH

Relevant characteristics of LEH (Terumo Co, Ltd, Tokyo, Japan) have been reported.11,12 Briefly, it is a liposome capsule measuring 230 nm in mean diameter, containing hemoglobin eluted from human RBC outdated for transfusion. The liposome capsule is coated...
with polyethylene glycol to reduce aggregation and capture by the reticulendothelial system to prolong the circulation half-life to 30 hours in rats. Inositol hexaphosphate is included for 2,3-diphosphoglycerate to adjust the O₂ affinity to P₅₀O₂=40 mm Hg in LEH (TRM-645), lower than that of rodent RBC (P₅₀O₂=30 mm Hg; Figure 1). Whereas LEH is considered to have a higher O₂ delivery than RBC under physiological conditions between 100 and 40 mm Hg, RBC is supposed to be more efficient in O₂ delivery than LEH under hypoxic conditions between 20 and 3 mm Hg.

Figure 1. O₂ dissociation characteristics of LEH (P₅₀O₂=40 mm Hg) and rodent RBC (P₅₀O₂=30 mm Hg). Whereas LEH is considered to have a higher O₂ delivery than RBC under physiological conditions between 100 and 40 mm Hg, RBC is supposed to be more efficient in O₂ delivery than LEH under hypoxic conditions between 20 and 3 mm Hg.

Flow Patterns of RBCs and Liposomes
The flow patterns were observed through a closed cranial window under a fluorescence microscopy and recorded to a high-speed video camera. The flow patterns of RBCs were observed after infusion of fluorescein-4-isothiocyanate–labeled RBCs, which emit 520 nm fluorescence in response to excitation by a mercury lamp light (wave length 490 nm). The flow patterns of liposomes were followed after infusing liposome encapsulated rhodamine-6G (0.5 mL), which emits 551 nm fluorescence in response to excitation by a halogen lamp (wave length 528 nm). These images were compared by switching fillers at the same frame from the cortical surface of the parietal lobe in rats.

Animal Treatment
All experiments were approved by the institutional review board of Tokai University School of Medicine and Hamamatsu Photonics. Rats received humane care as required.

Statistics
The TTC-demarcated areas or the values of rats were averaged for each group and compared among groups by ANOVA with repeated measures. P<0.05 was considered significant.

Results

Flow Patterns of Liposomes and RBCs
(supplemental movie file, available online at http://stroke.ahajournals.org)
Under fluorescence microscopy (Figure 3), fluorescein-4-isothiocyanate–labeled RBCs (left panel) were identified as individual dots flowing mainly through larger vessels at either high speed (artery) or low speed (vein), whereas fluorescent liposomes (right panel) were not individually identifiable but observed as a fluid filling all vessels, in a manner similar to that of plasma, regardless of diameter or type of vessels, arteries, capillaries, or veins.

LEH Behavior, O₂ Content, and Delivery
There was no fluctuation in systemic blood pressure before and after infusion of one of the solutions of an aliquot of 10 minutes at a slow speed (2.7 to 3.0 mL/10 minutes) to avoid acute volume load, and were compared with rats receiving no infusion (n=7). Experiments were repeated in the same way using LEH with serial 5-fold dilutions with saline so that the aliquot of infusate (10 mL/kg) would remain the same: 2 mL/kg (5-fold dilution; n=8), 0.4 mL/kg (25-fold dilution; n=8), and 0.08 mL/kg (125-fold dilution; n=6). After infusion, blood samples were taken (the “post” samples) and the infusion line was removed. Animals were placed back in cages in room air, with access to food and water ad libitum until evaluation of infarction.

Brain Damage Determination
Twenty-four hours later animals were euthanized by cervical vertebral dislocation under anesthesia after evaluating the MCA flow and obtaining a blood sample (the “1-day” sample). The brain was excised and sliced into 6 coronal slices of 2-mm thickness (2 slices anterior to and 4 slices posterior to the optical chiasm) with a brain slicer (Muromachi Kikai, Co, Ltd) and placed in 2,3,5-triphenyltetrazolium chloride (TTC) solution to demarcate the area of infarction. These 6 slices were placed in order, digitized, and integrated to calculate the infarcted and intact areas separately in the cortex and basal ganglia (Figure 2). After TTC staining, the brain slices were fixed with 4% formaldehyde and stained with hematoxylin-eosin, diamino-benzidine staining for iron, and immunohistochemical staining for human hemoglobin and microtubule-associated protein 2 (MAP2) by neuroanatomists (M.Y.) blind to the study protocol.

Animal Model
Male Sprague-Dawley rats of 10 to 12 weeks of age (270 to 300 grams, mean 283 grams; Japan SLC, Inc, Shizuoka, Japan) were used. Rats were anesthetized and maintained with 2% halothane in a mixture of 70% room air and 30% O₂ throughout the following procedure. After the insertion of an infusion line into the tail vein and blood sampling (“pre” sample), the scalp and temporal muscle were reflected and a subtemporal craniotomy was made. The main trunk of the left MCA was observed through the dura mater under an operative microscope through a window anterior to the foramen of the mandibular nerve. Photo-illumination by green light (wavelength, 540 nm) was achieved using a xenon lamp (model L4887; Hamamatsu Photonics) with a heat-absorbing filter and a green filter. After infusion of rose bengal (20 mg/kg), photo-illumination was delivered to the MCA through the dura for 10 minutes by a 3-mm optic fiber placed on the window in the skull base (photochemically induced thrombosis). After confirmation of thrombotic occlusion of the MCA, the incision was closed.

Infusion of Experimental Solutions
Immediately thereafter, rats received 10 mL/kg of LEH (n=7), saline (n=6), blood (n=6), or empty liposomes (n=5) infused over 10
mL/kg. Although hemoglobin levels were similar before and after onset of ischemia (Figure 4A), they became higher in transfused rats because of elimination of LEH from circulation at the time of euthanization, when MCA had been recanalized in all rats. Based on these hematocrit and LEH-crit values and their O₂ binding characteristics (Figure 1), the total blood O₂ content (Figure 4B) and plasma O₂ content (Figure 4C, less RBC-bound O₂, or sum of O₂ in the plasma and LEH) were calculated for each group of rats immediately after infusion and 24 hours later. Because of simple infusion, or top load, animals receiving transfusion or LEH had higher hemoglobin levels or total blood O₂ contents, regardless of the pO₂ or time after administration, than rats receiving vehicle (saline or empty liposomes). Among the former, total blood O₂ content (Figure 4B) was almost identical at the beginning as well as at the end of experiment, when transfused rats had a slightly higher O₂ content, reflecting elimination of LEH from circulation. In contrast, plasma O₂ content (Figure 4C), the sum of O₂ in plasma and LEH, was calculated to be higher in LEH-treated rats than animals receiving other solutions, which had the soluble O₂ alone. Although the difference appears striking (Figure 4C), the amount was less than one-tenth of the total blood O₂ content because of the absence of RBC.

**TTC Staining for Cerebral Infarction**

Typical examples of TTC staining (Figure 2) showed that LEH treatment mainly protected the parietal and frontal cortex (white arrows) than temporal cortex (black arrows) or basal ganglia, which were consistently affected in other control animals as well. Therefore, the integrated area of damage or infarction extent (Figure 5) was significantly suppressed in rats treated with LEH (P<0.05) as compared with rats receiving no infusion, vehicles, or transfusion in the cortex but not in basal ganglia, which had a similar degree of infarction regardless of treatment. The extent of infarction appeared to correlate with plasma O₂ content, but not with the total blood O₂ content (transfusion versus vehicles), the presence or absence of empty liposomes or volume infusion (vehicles versus no infusion). Stepwise dilution (Figure 5) revealed that 10 mL/kg or 2 mL/kg of LEH was comparably protective, whereas 0.4 mL/kg failed to yield significant protection (P=0.07) and 0.08 mL/kg had no effect as in other control solutions.

**Histopathology and LEH Staining**

Morphological changes were less severe in the LEH-treated animals (LEH 10 mL/kg) in hematoxylin-eosin staining (Figure 6 upper panels) as well as in MAP2 staining (Figure 6 lower panels). Although fluorescein-4-isothiocyanate–labeled RBCs and fluorescent liposomes were observed and compared by changing filters by a fluorescence microscopy through a closed cranial window from the cortical surface of the parietal lobe in a rat. Although fluorescein-4-isothiocyanate–labeled RBCs (left panel) were identified as individual dots flowing through mainly larger vessels, fluorescent liposomes (right panel) were not individually identifiable but were observed as a fluid filling all vessels regardless of size and type, pial arteries, and veins.
6 lower panels), in which cortical architecture was preserved in the parietal lobe (Figure 2, open arrows), than in the rats receiving saline (10 mL/kg), which displayed severe ischemic damage, including edema, loss of large pyramidal cells, swelling of small neurons, and spongiosis in the cortex. Immunohistochemical staining for MAP2 (Figure 7A) and human hemoglobin or iron for LEH (Figure 7B) revealed a dense expression of MAP2 with no LEH deposition in the intact hemisphere in contrast with a diffuse loss of MAP2 with intercellular LEH deposition in the ischemic hemisphere. LEH was limited to the vascular lumen in the intact tissue, detected in the intercellular space in the ischemic tissue, but not found in the vascular endothelial cells in any tissues, either intact or ischemic (Figure 7).

**Discussion**

Artificial O₂ carriers have been studied as blood substitutes mainly to reduce homologous blood transfusion.⁶⁻¹² The advantages of artificial O₂ carriers include a reduced, if not eliminated, risk of blood-borne infection and mismatch transfusion. Nonetheless, safety concerns and untoward effects still prevent clinical application, whereas short functional life may reduce their utility as blood substitutes.⁶⁻¹² Considering its perfusion characteristics deriving from its size and viscosity,¹¹,¹² we performed the current experiments to examine the hypothesis that LEH may better-perfuse collaterals and capillaries with plasma to reduce ischemic and reperfusion damage. The results suggest that LEH in a small dose (2 mL/kg) is effective in reducing the extent of infarction.
biochemically and morphologically in the cortex, but not in basal ganglia 24 hours after photochemically induced thrombosis occlusion of the MCA in a dose-dependent manner in the rat. The photochemically induced thrombosis model causes thrombotic occlusion first and reperfusion later as a result of thrombolysis,13,14 similar to the pathologic cascade occurring after clinical infarction, suggesting a use for LEH in human brain ischemia and reperfusion. Its relatively short intravascular half-life ($T_{1/2} \approx 30$ hours)12 may make it acceptable or even advantageous for short-term use as a therapeutic system for targeted O$_2$ delivery to ischemic tissues, or “O$_2$ therapeutics.”

Because ischemia impairs the active transport of electrolytes,1,16 water shifts from the intravascular compartment to the tissue, causing an increase in blood viscosity and tissue pressure,16 both of which impair microcirculation. When intracranial pressure rises as a result,17 small particles may prevent a collapse in the microcirculation.2 Nonetheless, empty liposomes failed to be protective, underscoring the importance of hemoglobin or O$_2$-carrying capacity. Although the O$_2$ dissociation characteristics are quite different (Figure 1), the total O$_2$ content and average O$_2$ delivery were mostly higher in transfused rats, which nonetheless displayed no protection, suggesting that hemoglobin or O$_2$ transport in the form of RBCs failed to exert the protective effect. Recent studies3,4 have suggested that plasma perfuse capillaries not only to the ischemic penumbra but also to the core of ischemia even when RBC flow is severely reduced and inhomogeneous,18 providing a rationale for LEHs, but not RBCs, to perfuse, deliver O$_2$, and thereby to be protective in brain ischemia. In this regard the effect of LEH may be similar to that of hyperbaric oxygen therapy,5 which uses plasma instead of RBCs to deliver O$_2$. The advantages of hemoglobin-based O$_2$ carriers such as LEH$^{11,12}$ and cell-free hemoglobin$^8$–10 over hyperbaric oxygen therapy$^5$ or perfluorochemicals$^6$–7 include sigmoid O$_2$ dissociation characteristics, which allow room air respiration to be clinically efficient, as in the present study. In comparison to cell-free hemoglobin$^8$–10 LEH is structurally similar to RBC, encapsulated and large enough to prevent renal clearance, extravasation, or hypertension response, which might have contributed to the fact that 2 mL/kg of LEH was protective in

![Figure 5. TTC-defined area of infarction compared among rats receiving no infusion, saline, transfusion, empty liposomes, and LEH at one of the following concentrations: 10 mL/kg, 2 mL/kg, 0.4 mL/kg, or 0.08 mL/kg in the cortex and basal ganglia. The number near the bottom of each bar indicates the number of rats in each group.](image)

![Figure 6. Hematoxylin-eosin staining (upper panels) and immunohistochemical staining for MAP2 (bottom panels) in the parietal cortex of the ischemic hemisphere in rats treated with saline (saline, left panels) and 10 mL/kg of LEH (LEH, right panels). Morphological changes were less severe in the LEH-treated animals, in which cortical architecture was preserved, than in the saline-treated rat with severe ischemic damage, including edema, loss of large pyramidal cells, swelling of small neurons, and spongiosis in the cortex.](image)
contrast to polymerized hemoglobin,\textsuperscript{8,9} which required exchange transfusion in order to infuse 38 mL/kg, more than one-half of the total blood volume in mice (72 mL/kg). We also found LEH to be effective in much smaller doses in brain ischemia in primates (0.4 mL/kg),\textsuperscript{19} in wound healing (0.4 mL/kg),\textsuperscript{20} tumor oxygenation (5 mL/kg),\textsuperscript{21} and in myocardial ischemia (10 mL/kg),\textsuperscript{22} all known as indicators for hyperbaric O\textsubscript{2} therapy, suggesting that effects of LEH are basically related to its O\textsubscript{2}-carrying capacity and particle size small enough to flow like plasma rather than RBC.

Artificial O\textsubscript{2} carriers are not only delivering O\textsubscript{2} but also acquiring O\textsubscript{2} in the lungs as long as they remain in circulation. The amount of O\textsubscript{2} delivered at one time by the infiltrating LEH is considered to be so small that it may not be sufficient either to save tissue, as in the basal ganglia, or to yield O\textsubscript{2} radicals in severe hypoxia.\textsuperscript{23} However, multiple recirculation may make the total amount of O\textsubscript{2} delivery significant and crucial for ischemic neurons in peril of death or apoptosis and make the reperfusion injury less severe. Such multiple recirculation with plasma may account for the cortical protection with little tissue deposition and the extended dose-response relationship\textsuperscript{24}: LEH was highly protective at 2 mL/kg, almost effective at 0.4 mL/kg, and not protective at 0.08 mL/kg. The absence of added protection with the use of 10 mL/kg over 2 mL/kg may suggest that 10 mL/kg is transporting more O\textsubscript{2} than the ischemic tissue can handle and reverse protection.\textsuperscript{23} Benefit of LEH may not correlate with the total O\textsubscript{2} content or delivery, but with an amount of plasma O\textsubscript{2} much smaller than we thought, which is necessary for the ischemic neural tissue to survive before reperfusion. From the current results, however, the involvement of other functions of hemoglobin, including bicarbonate buffering, nitric oxide regulation, and leukocyte inflammatory response suppression, may not be ruled out.

Pathologic studies consistently showed less edema and ischemic damage in LEH-treated rats as compared with control animals. Immunohistochemical staining for MAP2, which is highly concentrated in dendrites and serves as an
indicator of acute brain injury,15 revealed better protection in the cortex than in basal ganglia, which in contrast had accumulated LEH. Basal ganglia were located at the core of ischemia immediately distal to photochemically induced thrombosis occlusion of MCA with little protection regardless of treatment,13,14 which in turn suggests that rats were subjected to similar degrees of ischemia. The protection by LEH was largely limited to the parietal and frontal cortex (Figure 2) located at the periphery of the ischemia, suggesting salvage by collateral circulation of LEH2,3 or developmental differences in the structure and blood supply.25 Overlap with individual research funds, there is no conflict of interest.

Scientist (neuroanatomy) at Osaka Prefecture University, where all encapsulated hemoglobin tested in this study. M.Y. is a Research by Terumo Co Ltd, which developed and supplied liposome-animal experiments were performed. Y.O. is a Researcher employed by Hamamatsu Photonics, where all H.T. are Researchers employed by Hamamatsu Photonics, where all School of Medicine (Institute 1) and organized this study. D.F. and

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Science, and Technology, Tokyo, Japan (to A.T.K.); New Energy Development Technology, Tokyo, Japan (to A.T.K.); CREST, Japan Science and Technology Agency (J.S.T.), Japan; and the Japanese Ministry of Health, Labour and Welfare (to A.T.K.).

A.T.K. and M.H. are Clinicians/Scientists at the Tokai University School of Medical Institute 1) and organized this study. D.F. and H.T. are Researchers employed by Hamamatsu Photonics, where all animal experiments were performed. Y.O. is a Researcher employed by and supplied liposome-encapsulated hemoglobin tested in this study. M.Y. is a Research Scientist (neuroanatomy) at Osaka Prefecture University, where all the morphological studies were performed. Because all authors had individual research funds, there is no conflict of interest.

Disclosures

A.T.K. and M.H. are Clinicians/Scientists at the Tokai University School of Medicine (Institute 1) and organized this study. D.F. and H.T. are Researchers employed by Hamamatsu Photonics, where all animal experiments were performed. Y.O. is a Researcher employed by and supplied liposome-encapsulated hemoglobin tested in this study. M.Y. is a Research Scientist (neuroanatomy) at Osaka Prefecture University, where all the morphological studies were performed. Because all authors had individual research funds, there is no conflict of interest.

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Stroke. 2007;38:1626-1632; originally published online March 29, 2007; doi: 10.1161/STROKEAHA.106.467290

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/38/5/1626

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