Superior Microvascular Perfusion of Infused Liposome-Encapsulated Hemoglobin Prior to Reductions in Infarctions after Transient Focal Cerebral Ischemia

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Background: The development of cerebral infarction after transient ischemia is attributed to postischemic delayed hypoperfusion in the microvascular region. In the present study, we assessed the microvascular perfusion capacity of infused liposome-encapsulated hemoglobin (LEH) in a therapeutic approach for transient middle cerebral artery occlusion (tMCAO). *Methods:* Two-hour middle cerebral artery occlusion rats were immediately subjected to intra-arterial infusion of LEH (LEH group) or saline (vehicle group) or no treatment (control group), and then to recanalization. Neurological findings, infarct and edema progression, microvascular endothelial dysfunction, and inflammatory reactions were compared between the 3 groups after 24 hours of reperfusion. Microvascular perfusion in the early phase of reperfusion was evaluated by hemoglobin immunohistochemistry and transmission electron microscopy. *Results:* The LEH group achieved significantly better results in all items evaluated than the other groups. Hemoglobin immunohistochemistry revealed that the number of hemoglobin-positive microvessels was significantly greater in the LEH group than in the other groups ($P < .01$), with microvascular perfusion being more likely in narrow microvessels (≤5 μm in diameter). An electron microscopic examination revealed that microvessels in the control group were compressed and narrowed by swollen astrocyte end-feet, whereas those in the LEH group had a less deformed appearance and contained LEH particles and erythrocytes. *Conclusion:* The results of the present study demonstrated that the infusion of LEH reduced infarctions after tMCAO with more hemoglobin-positive and less deformed microvessels at the early phase of reperfusion, suggesting that the superiority of the microvascular perfusion of LEH mediates its neuroprotective effects. **Key Words:** Infarction—transient focal cerebral ischemia—microvessels—neuroprotection.

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Stroke is a major cause of death and disability in many developed nations. The recent establishment of revascularization therapies for acute ischemic stroke may result in more favorable outcomes than those previously achieved. However, ischemia-reperfusion (I/R) after revascularization therapies sometimes causes tissue damage that develops after vascular recanalization due to the pre-existence of a prolonged and severe ischemic period.[1] Based on animal experimental models, the development of infarction after transient ischemia is assumed to be due to postischemic delayed hypoperfusion in the microvascular region. Decreased capillary flow with capillary diameter reductions was demonstrated in delayed postischemic hypoperfusion using a video microscopic analysis.[2] In addition, previous studies using electron microscopy reported that I/R induced astrocytic end-foot swelling, which transiently compressed and narrowed adjacent microvessels from a couple of hours to several hours after reperfusion.[3,4] Therefore, microvascular narrowing may contribute to postischemic delayed hypoperfusion.

Liposome-encapsulated hemoglobin (LEH) was originally developed as a blood substitute using technologies for the encapsulation of concentrated hemoglobin with liposomes in order to overcome issues associated with blood transfusion.[5] The mean diameter of LEH ranges between 200 and 250 nm (1/40th the size of erythrocytes), and this is expected to be advantageous for perfusion into microvessels. Therefore, LEH has been examined as a therapeutic agent for ischemic lesions with perfusion disturbances. Previous studies demonstrated the therapeutic effects of LEH using animal brain ischemic models.[6-12] Another advantage of LEH as artificial blood is that it does not contain inflammatory cells. As neutrophils cause inflammatory reactions, which lead to tissue damage after transient ischemia, namely I/R injury, the suppression of the neutrophil function may be employed as a therapeutic strategy for I/R injury.[13] The selective arterial infusion of LEH transfers oxygen to affected regions with reductions in the influx of neutrophils. In our previous study, we selectively infused LEH into the recanalized artery of a rat transient middle cerebral artery occlusion (tMCAO) model in order to ameliorate tissue damage induced by neutrophil-mediated inflammatory reactions. The selective infusion of LEH for 2 hours reduced the influx of autologous blood cells and ameliorated tissue damage in the reperfused area.[14] In the present study, we focused on the microvascular perfusion capacity of LEH in order to investigate the neuroprotective effects of LEH in a rat tMCAO model. LEH was administrated from the recanalized internal carotid artery for a short-term period in order to confirm whether infused LEH is superior to autologous erythrocytes in terms of microvascular perfusion and if LEH superior perfusion precedes reductions in infarctions after tMCAO.

Material and Methods

Animals

All animal experiments were approved by the Animal Studies Ethical Committee at the Hokkaido University Graduate School of Medicine. All procedures used in this study were performed in accordance with the institutional guidelines for animal experiments.

LEH

LEH was developed, manufactured, and provided by Terumo Corporation (Tokyo, Japan). The relevant characteristics of LEH have been reported.[5] LEH contains inositol hexaphosphate, which is used as an allosteric effector to modulate the oxygen affinity of Hb over that of usual erythrocytes. The average size of liposomes ranges between 200 and 250 nm, which is 1/40th the size of erythrocytes. The liposomal surface is modified polyethylene glycol to increase LEH stability during storage and use in blood by preventing aggregation. LEH was suspended in saline at a hemoglobin concentration of 6 g/dL. LEH was oxygenated to a PaO_2 of 110 ± 10 mmHg by mixing with room air prior to use.

Middle cerebral artery occlusion (MCAO) Model and Experimental Groups

Male Sprague-Dawley rats (CLEA Japan, Inc., Tokyo, Japan) weighing between 260 and 300 g were anesthetized using 4.0% isoflurane in N_2O:O_2 (70:30) and maintained with 2.0% isoflurane in N_2O:O_2 (70:30) through a facial mask. Focal cerebral ischemia was induced by right MCAO using a modified method of intraluminal suture occlusion.[14,15] Regional cerebral blood flow was measured using laser Doppler flowmetry (OMEGA FLOW FLO-C1; OMEGAWAVE, Tokyo, Japan) before and after MCAO in order to confirm successful MCAO. The location of the flowmetry probe to measure CBF was 2 mm posterior and 5 mm lateral to the bregma. Rats that did not show a regional blood-flow reduction of 30% of the preischemic baseline were excluded from further experimentation. Rats subjected to 2-hour MCAO were divided into 3 groups: (1) an LEH group infused with LEH (20 mL/kg) through a catheter for 15 minutes via the ICA; (2) a vehicle group infused with saline in the same manner as the LEH group; and (3) a control group subjected to recanalization only without the infusion. Infused LEH and saline were warmed to 37°C before infusion to avoid hypothermic neuroprotective effects due to low-temperature fluid infusion. Rats were killed 24 hours after reperfusion, and brains were collected to examine pathological changes.

A total of 116 rats were enrolled in the experiments, and 88 rats (75.9%) were included for data analysis. The reasons for the exclusion of 28 rats were lethal intraoperative complications (n = 6), insufficient CBF reductions (n = 7), insufficient neurological deterioration (n = 11), and