Decreased Damage From Transient Focal Cerebral Ischemia by Transfusion of Zero-Link Hemoglobin Polymers in Mouse

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Background and Purpose—Transfusion of large polymers of hemoglobin avoids the peripheral extravasation and hypertension associated with crosslinked tetrameric hemoglobin transfusion and may be more effective in rescuing brain from focal ischemia. Effects of transfusion of high-oxygen-affinity hemoglobin polymers of different weight ranges were determined.

Methods—Hypervolemic exchange transfusion was performed during 2 hours of middle cerebral artery occlusion in mice.

Results—Compared to transfusion with a 5% albumin solution or no transfusion, infarct volume was reduced 40% by transfusion of a 6% solution containing hemoglobin polymers in the nominal range 500 to 14 000 kDa. Infarct volume was not significantly reduced by transfusion of a lower concentration of 2% to 3% of this size range of polymers, 6% hemoglobin solutions without removal of polymers, or crosslinked hemoglobin tetramers with normal oxygen affinity. Exchange transfusion with the 6% solution of the 500 to 14 000 kDa hemoglobin polymers did not improve the distribution of cerebral blood flow during focal ischemia and, in mice without ischemia, did not affect flow to brain or other major organs.

Conclusion—An intermediate size range of polymerized bovine hemoglobin possessing high oxygen affinity appears optimal for rescuing mouse brain from transient focal cerebral ischemia. A minimum concentration of a 6% solution is required, the rescue is superior to that obtained with crosslinked tetrameric hemoglobin possessing normal oxygen affinity, and tissue salvage is not associated with increased blood flow. This polymer solution avoids the adverse effects of severe renal and splanchnic vasoconstriction seen with crosslinked tetrameric hemoglobin. (Stroke. 2009;40:278-284.)

Key Words: blood substitutes ■ cerebral blood flow ■ hemoglobin-based oxygen carrier ■ middle cerebral artery occlusion ■ stroke

Decreased blood viscosity associated with hemodilution has been considered as a strategy to increase cerebral blood flow (CBF) through collateral arteries and limit the spread of focal cerebral ischemic injury. However, early clinical trials of hemodilution failed to demonstrate improved outcome.1,2 Reasons for the failure include the limited degree of hemodilution, relatively small decreases in blood viscosity, the decrease in O2 carrying capacity, and the need to prevent hypovolemia.3,4

Exchange transfusion with a hemoglobin-based oxygen carrier (HBOC) provides a means to decrease hematocrit to a greater extent without large decreases in O2 carrying capacity. The first generation of HBOCs used hemoglobin (Hb) molecules in which the tetrameric structure was stabilized by forming covalent crosslinks between the subunits. Hypervolemic exchange transfusion of diaspirin αα-crosslinked tetrameric Hb before or during transient middle cerebral artery occlusion (MCAO) in rats was found to reduce infarct size.5,6 However, efficacy of this product could not be demonstrated in a clinical stroke trial possibly because of the delay in instituting the transfusion and adverse side effects.7,8 One of the side effects of crosslinked tetrameric Hb is extravasation in peripheral vascular beds, where Hb can scavenge nitric oxide, increase endothelin production, and produce vasoconstriction.9–11 Strategies to limit extravasation in the second generation of HBOCs include encapsulation in liposomes, conjugation with polyethylene glycol, and formation of large polymers.12,13 Glutaraldehyde has been used as a polymeriza-
tion reagent in products undergoing clinical testing in non-stroke trials, but the resulting polymers are heterogeneous in size and small polymers may still extravasate.

Using recombinant expression systems, polymers of engineered Hb could be generated with high O₂ affinity. Interestingly, transfusion of recombinant Hb polymers possessing P₅₀ (pO₂ at 50% oxyhemoglobin saturation) of 3 and 17 mm Hg decreased infarct volume after transient MCAO in mice. This finding raised the possibility that an HBCO with a P₅₀ intermediate between the P₅₀ of red blood cell (RBC) Hb and the P₅₀ of ischemic tissue may facilitate O₂ transport. However, one constraint of current recombinant technology is the limited amount of protein that can be generated. Chemically modified bovine Hb can be produced more readily in large quantities. We have reported on the production of large polymers of bovine Hb in which chemically stabilized tetramers form amide covalent bonds without retaining the polymerization reagent. This polymer, termed zero-link polymers, was found to have a nominal molecular weight (MW) of 20 MDa and a P₅₀ of 4 mm Hg. Moreover, the polymer did not extravasate in renal lymph or produce arterial hypertension, in contrast to the extravasation and hypertension observed with tetrameric crosslinked Hb.

The purpose of the present study was 4-fold. First, because the polymers produced by this process have a wide range of MW, the effect on infarct volume of removing low and high MW components from a solution that was exchange transfused during MCAO was determined. Second, the dose response of the optimal MW solution on infarct volume was determined and compared to the effect of crosslinked tetrameric Hb. Third, the effect of exchange transfusion of ZL-HbBv on cerebral and peripheral blood flow was evaluated without induction of MCAO to determine if this polymerized hemoglobin product produces regionally selective vasocostriction. Fourth, the effect of exchange transfusion of polymerized Hb during MCAO on regional CBF distribution was determined.

Materials and Methods

Hemoglobin Preparation

Synthesis of ZL-HbBv has been described. In brief, purified bovine Hb that had first undergone tetrameric stabilization by crosslinking the subunits was then incubated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and N-hydroxysulfosuccinimide to form stable pseudopeptide bonds between side chain carboxylates and ε-amino groups of lysyl residues of adjacent Hb molecules. After stopping the reaction with ethylene-diamine, pasteurization, and dialysis against a phosphate buffer, the resulting solution has heterogeneous Hb molecules ranging in MW from residual 64 kDa and dialysis against a phosphate buffer, the resulting solution has heterogeneous Hb molecules ranging in MW from residual 64 kDa and the P₅₀ of ischemic tissue may facilitate O₂ transport. However, one constraint of current recombinant technology is the limited amount of protein that can be generated. Chemically modified bovine Hb can be produced more readily in large quantities. We have reported on the production of large polymers of bovine Hb in which chemically stabilized tetramers form amide covalent bonds without retaining the polymerization reagent. This polymer, termed zero-link polymers, was found to have a nominal molecular weight (MW) of 20 MDa and a P₅₀ of 4 mm Hg. Moreover, the polymer did not extravasate in renal lymph or produce arterial hypertension, in contrast to the extravasation and hypertension observed with tetrameric crosslinked Hb.

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MCAO All procedures on mice were approved by the Johns Hopkins University Animal Care and Use Committee and conformed to the guidelines of the National Institutes of Health. Focal cerebral ischemia was produced in C57Bl/6 male mice (Charles River Laboratories; Wilmington, Del) by the intraluminal filament technique. Mice were anesthetized with 1.5% halothane via face mask, rectal temperature was maintained with a heating lamp, and a femoral artery was catheterized. The right common and external carotid arteries were isolated, and a 6-0 monofilament with a blunt tip was advanced from the cut stump of the external carotid artery into the internal carotid artery. The filament was inserted 6 mm past the junction of the pterygopalatine artery to produce MCAO for 2 hours. After closing the incisions with suture, the halothane concentration was decreased to 0.8%, which was adequate to prevent spontaneous limb movement in the absence of surgical stimulation. In most groups, an exchange transfusion commenced at 10 minutes of MCAO and ended at 30 minutes of MCAO. At 45 minutes of MCAO, an arterial blood sample was obtained and the volume was replaced by the same solution used for the exchange transfusion. The total volume infused was 950 μL and the total amount withdrawn was 700 μL. The withdrawal and infusion through a single femoral artery catheter were alternated in 4 incremental steps. The exchange transfusion was moderately hypervolemic to assure good perfusion pressure during MCAO.

In survival experiments, anesthesia was discontinued at 1 hour of MCAO, and neurological deficits consisting of circling behavior and weak contralateral limb function were assessed to assure adequate ischemia. Reperfusion was established at 2 hours by withdrawal of the filament during a brief period of anesthesia. Infarct volume was measured at 24 hours of reperfusion by vital dye staining with 2,3,5-triphenyltetrazolium staining and image analysis of both sides of 5 coronal slabs with adjustment for swelling. In nonsurvival experiments, regional blood flow was measured either without MCAO or at 90 minutes of MCAO under 0.8% halothane anesthesia.

Blood Flow Measurements

Regional blood flow was measured by autoradiography using [¹⁴C]-iodoantipyrine as a tracer. A dose of 4 μCi of [¹⁴C]-iodoantipyrine was infused through a femoral vein catheter at a rate of 108 μL/min over 45 seconds, while femoral arterial blood was sampled over 5-second intervals to obtain the arterial input function. At 45 seconds of infusion, the tissue was rapidly harvested, frozen, and later cut into 20-μm sections, which were mounted on slides apposed to film. In nonischemic mice, tissue was analyzed from cerebral cortex, striatum, heart, renal cortex, renal medulla, small intestine, and skeletal muscle from noncatheterized limbs. For intraischemic CBF distribution, tissue was analyzed from 6 sections at each of 6 coronal levels spaced 1 mm apart from +2 to −3 mm from bregma. For each hemisphere, the volume of tissue with blood flow in different ranges of 10 mL/min per 100-gram increments was calculated.

Experimental Design

In the first experiment, infarct volume was measured in groups that had undergone exchange transfusion with either 5% human serum albumin (n=5), 5% human serum albumin plus infusion of phenylephrine during MCAO (n=8), 6% polymerized Hb with low MW species removed (n=5), 6% polymerized Hb with high MW species removed (n=5), and 6% polymerized Hb with both low- and high-MW species removed (n=5). In the second experiment, infarct volume was measured in groups that had undergone either no exchange transfusion (n=12) or exchange transfusion with either 5% human serum albumin (n=11), 6% sebacoyl crosslinked Hb (n=9), 2% to 3% ZL-HbBv (low- and high-MW species removed; n=11), and 6% ZL-HbBv (n=10). In the third experiment, blood flow to brain and peripheral organs was measured in nonischemic groups.
with no transfusion (n=6) or after exchange transfusion with ZL-HbBv (n=7). In the fourth experiment, the volume of brain tissue with different ranges of CBF was measured during MCAO in groups with no transfusion (n=5) or after exchange transfusion with ZL-HbBv (n=5).

Statistical Analysis
In the first 2 experiments, infarct volume was compared among groups by ANOVA and the Newman-Keuls multiple range test. In the third experiment, blood flow to each organ was compared between the control group and ZL-HbBv–transfused group by t test. In the fourth experiment, 2-way ANOVA was performed with the transfusion group as a between-subject factor and blood flow range as a within-subject factor. Data are presented as mean±SE.

Results
Effect of Different MW Polymers on Infarct Volume
A hypervolemic exchange transfusion performed with albumin or various MW fractions of Hb polymers during MCAO produced similar decreases in arterial hematocrit (Figure 1). The corresponding decrease in arterial Hb concentration was less in the groups receiving Hb polymers. The hypervolemic exchange transfusion produced a small increase in mean arterial blood pressure (MABP) in all groups, and MABP was not significantly different among groups after the transfusion. However, because the increase in MABP in the 3 groups receiving the Hb polymers (7–12 mm Hg) tended to be greater than in the albumin-transfused group (5 mm Hg), an additional group received an infusion of phenylephrine after transfusion of albumin to produce a 12-mm Hg increase in MABP for the remainder of MCAO.

Infarct volume was not significantly different between the albumin-transfused groups with and without phenylephrine infusion (Figure 1). In addition, groups transfused with the Hb solutions in which either the low- or high-MW species were removed had large infarcts that were not significantly different from either of the albumin-transfused groups. However, the group transfused with the Hb solution with both low- and high-MW species removed had a 39% decrease in infarct volume. Therefore, ZL-HbBv with only intermediate size polymers was used in subsequent experiments.

Effect of Polymeric Hb Concentration and Comparison With Tetrameric Hb on Infarct Size
Infarct volume in a group transfused with the 5% albumin solution was not significantly different from a group that did not undergo exchange transfusion (Figure 2). Exchange transfusion with a solution of 2% to 3% ZL-HbBv and 6% ZL-HbBv decreased hematocrit to a similar extent. As expected, arterial blood Hb concentration after exchange transfusion with the 2% to 3% ZL-HbBv solution was intermediate between that obtained with the 6% ZL-HbBv and 5% albumin solutions (Figure 2). Infarct volume in the group
transfused with the 2% to 3% ZL-HbBv solution averaged 11% less than the albumin-transfused group, but the difference was not significant. With exchange transfusion of the 6% ZL-HbBv solution, infarct volume was decreased by 40% from the albumin group and 37% from the nontransfused group, and infarct volume was significantly less than the value in the group transfused with the 2% to 3% solution.

Exchange transfusion of the 6% sebacoyl-crosslinked Hb resulted in a total Hb concentration that was similar to the 6% ZL-HbBv solution (Figure 2). However, infarct volume in the group transfused with 6% sebacoyl-crosslinked Hb was greater than that in the 6% ZL-HbBv–transfused group and similar to that in the 2% to 3% ZL-HbBv–transfused group. MABP after transfusion of sebacoyl-crosslinked Hb was greater than after transfusion of albumin, whereas MABP in the groups transfused with ZL-HbBv was not significantly different from the albumin-transfused group. The plasma half-life in the mouse of ZL-HbBv (217 min) and sebacoyl-crosslinked Hb (168 min) exceeded that of unmodified HbA (34 min).

**Regional Blood Flow**

In mice not undergoing MCAO, CBF in cerebral cortex and striatum was similar in control and ZL-HbBv–transfused groups (Figure 3). Likewise, no significant differences between the control and ZL-HbBv–transfused groups was present in blood flow to heart, renal cortex, renal medulla, small intestine, or skeletal muscle.

**Effect of Polymeric Hb on Intraischemic Blood Flow Distribution**

In mice undergoing MCAO, a histogram of the volume of tissue in ipsilateral hemisphere between +2 and −3 mm from...
Hb transfusion had no significant effect on blood flow distribution. The major findings of this study are: (1) that transfusion of polymers of chemically modified bovine Hb during MCAO produced reductions in infarct size that were comparable to the recombinant Hb possessing a P50 of 4 mm Hg; (2) that crosslinked tetrameric Hb with a P50 comparable to RBC Hb P50 was less effective because of reduced infarct volume despite the low P50 and consequent increases in perivascular pO2.13 However, during cerebral ischemia, increasing the concentration of crosslinked Hb tetramers was found to produce additional decrements in infarct volume.5 We also found that a 6% solution of Hb polymers produced a more significant reduction in infarct volume than 2% to 3% infused concentrations. This finding is also consistent with previous work that used recombinant Hb polymers in the mouse.17 Increasing the concentration could have a beneficial effect by delivering more O2 immediately after transfusion and by extending the duration of effective plasma concentrations. The plasma half-life of 3.6 hours for ZL-HbBv in the mouse was shorter than the 7-hour value in rat and 10-hour value in cat.18 These results are consistent with the effect of body size on plasma half-life of cell-free Hb and the long-half life exceeding 24 hours of other Hb molecules in humans.21,22 Future work is required to determine if higher concentrations or more prolonged infusions provide additional rescue from ischemia.

Exchange transfusion of ZL-HbBv in nonischemic cat produced constriction of pial arterioles with no change in CBF.23 The constriction could be reversed to dilation if plasma viscosity was simultaneously increased. The constriction response appeared to adjust for the decrease in whole blood viscosity associated with a decrease in hematocrit and to keep CBF and O2 transport constant. The present finding that CBF in nonischemic mouse after ZL-HbBv transfusion was similar to control mice also implies that moderate cerebrovascular constriction occurred to offset the decrease in blood viscosity associated with a decrease in hematocrit. Furthermore, the pial arteriolar constriction response seen after exchange transfusion with ZL-HbBv in rat was blocked by a 20-HETE synthesis inhibitor and not by a nitric oxide synthase inhibitor.24 Thus, O2-dependent 20-HETE synthesis from arachidonic acid rather than scavenging of nitric oxide by Hb appears to be responsible for the constriction response to decreased blood viscosity at the near-normal O2 carrying capacity provided by cell-free Hb. In the case of MCAO, tissue hypoxia would be expected to block O2-dependent synthesis of 20-HETE from arachidonic acid and permit vasodilation. Hence, the decrease in hematocrit associated with ZL-HbBv exchange transfusion would be expected to result in a higher CBF compared to the control group if arterioles were maximally dilated in both groups. However, the CBF distribution during MCAO was not different between groups and the trend was in the direction of more tissue with low CBF in the ZL-HbBv-transfused group despite a lower hematocrit. Hence, we cannot exclude that vasodilation after MCAO was diminished in the ZL-HbBv–transfused group. Perhaps increased precapillary O2 loss and increased oxygenation of collateral arteries by ZL-HbBv limited vasodilation.

Salvage of tissue injury by this HBOC without a significant increase in intras ischemic CBF implies that improved O2 delivery was enhanced by increasing the effective microcirculatory surface area for diffusion or by facilitating O2 unloading from RBCs. In the former case, the effective surface area could be improved by an HBOC interspersed between RBCs moving at a slow velocity in individual capillaries or by perfusing capillaries that are poorly perfused by RBCs, thereby improving the homogeneity of O2 flux. The case for facilitated O2 transport is based on the knowledge that plasma normally represents a significant resistance for O2 diffusion from the RBC to the endothelium and that cell-free Hb may...
facilitate this transport.\textsuperscript{26,27} In this regard, it is of interest that removing the high-MW polymers improved the salvage of ischemic tissue. Perhaps the low mobility of very large polymers restricts the facilitation of O\textsubscript{2} diffusion in the plasma. A greater number of intermediate size polymer molecules may be advantageous for facilitating O\textsubscript{2} transport compared to a fewer number of large polymer molecules possessing the same overall concentration of heme. Another consideration not addressed in this study is that Hb polymers may improve bulk flow or the homogeneity of O\textsubscript{2} flux at the capillary level after reperfusion when the polymers are still present in the plasma.

Also of interest is the observation that removing the high-MW polymers without removing the low-MW polymers and crosslinked tetramers failed to reduce infarct volume. Indeed, infarct volume was comparable to the large infarction obtained with a sebacoyl crosslinked tetrameric Hb. These results suggest that residual crosslinked tetramers may exert an adverse effect that counteracts any beneficial effect on O\textsubscript{2} diffusion. Transfusion of sebacoyl crosslinked Hb was previously found not to reduce acute injury from permanent MCAO in cat.\textsuperscript{28} Although fumaryl αααα-crosslinked Hb reduced infarct volume in rat,\textsuperscript{6} a clinical trial with this product was stopped because of safety concerns. Crosslinked tetrameric Hbs produce peripheral vasoconstriction and the constriction is most marked in renal and splanchnic bed possessing large pores in the endothelium.\textsuperscript{10,11,29} In contrast to ZL-HbBv, the sebacoyl crosslinked Hb accumulates in renal lymph.\textsuperscript{18} The lack of effect of ZL-HbBv on blood flow to kidney and small intestine in the present study is also in contrast to the decreased blood flow seen after crosslinked tetrameric Hb transfusion.\textsuperscript{29} This peripheral vasoconstriction appears to be related to extravasation, scavenging of nitric oxide, and production of endothelin.\textsuperscript{10,11,29} Patients receiving the crosslinked tetrameric Hb after stroke were reported to have increased plasma endothelin levels,\textsuperscript{8} which may have reduced intraischemic CBF. Peripherally generated endothelin induced by extravasated crosslinked tetrameric Hb could have counteracted the benefit of this HBOC. Extravasation of crosslinked tetramers might also occur in ischemic brain and depend on the timing of transfusion relative to the delayed disruption of the blood–brain barrier. Polymerization would presumably reduce extravasation across a leaky blood–brain barrier.

The ineffectiveness of crosslinked tetrameric Hb in a clinical trial\textsuperscript{7} led many to abandon the approach of delivering O\textsubscript{2} to ischemic tissue by an HBOC. However, the strategy of Hb polymerization, along with other strategies of encapsulation or pegylation, may still hold promise as a therapy for delivering O\textsubscript{2} to ischemic brain tissue. The present findings show that careful assessment of a polymeric Hb solution that does not retain the polymerization agent has the potential of ameliorating short-term stroke injury. It should be noted that the present study was designed as an initial screen to evaluate different ranges of polymer size and concentrations on short-term outcome. To detect small differences among the various solutions, rescue was maximized by starting the transfusion soon after MCAO rather than using a clinically relevant delay. Future work will be required to thoroughly evaluate the optimal transfusion protocol, therapeutic window, and long-term histology and behavioral outcomes to satisfy the recommended criteria for pharmacological interventions in preclinical studies.\textsuperscript{30}

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**Disclosures**

E.B. and the University of Maryland are holders of a patent on the zero-link bovine hemoglobin polymer used in this study. R.C.K. and H.K. were paid consultants to Oxyvita Inc., holder of the licensing rights to the zero-link bovine hemoglobin polymer. The terms of this arrangement were managed by the Johns Hopkins University in accordance with its conflict of interest policies.

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