ORIGINAL RESEARCH

Transfusion of Polynitroxylated Pegylated Hemoglobin Stabilizes Pial Arterial Dilation and Decreases Infarct Volume After Transient Middle Cerebral Artery Occlusion

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***Background*—**Polynitroxylation of hemoglobin confers superoxide dismutase–mimetic and peroxidase activity and may protect from reperfusion injury in addition to facilitating oxygen transport. We determined whether transfusion of polynitroxylated PEGylated hemoglobin (PNPH) is protective in the rat filament model of 2 hours of middle cerebral artery occlusion (MCAO).

***Methods and Results*—**Transfusion of 10 mL/kg of PNPH at 20 minutes of MCAO reduced infarct volume by over 70% (n=10). To determine whether PNPH might act by promoting vasodilation, pial arteriolar diameter in the distal MCA border region was measured in closed cranial windows. With no transfusion, MCAO induced an initial dilation (36±2% ±SE) that subsided by 2 hours (5±4%; n=8). With PNPH transfusion at 20 minutes of MCAO, the initial dilation (31±3%) was better maintained at 2 hours (21±4%; n=7; $P<0.02$). Delaying PNPH transfusion until 90 minutes of MCAO increased perfusion in the border region from 48±6% of the preischemic baseline to 67±8% (n=8; $P<0.005$). The effect of PNPH transfusion after reperfusion was also tested. Compared with the control median hemispheric infarct volume of 22% (13% to 34% interquartiles; n=15), infarct volume was reduced to 7% (3% to 13%; n=14 $P<0.05$) when PNPH was transfused at 4 hours after MCAO (2 hours of reperfusion) but not significantly when transfused at 6 hours (8%; 3% to 35%; n=14) or at 8 hours (12%; 10% to 25%; n=14) after MCAO.

***Conclusions*—**PNPH transfusion has a significant therapeutic window for protection during and after transient MCAO and may act, in part, by stabilizing vascular function and improving collateral blood flow. **(*J Am Heart Assoc*. 2017;6:e006505. Doi: 10.1161/JAHA.117.006505.)**

Key Words: cerebral blood flow • hemoglobin • ischemic stroke • pial vessels • rats

Following occlusion of the middle cerebral artery (MCA), viability of neurons in the ischemic border region can be sustained for a finite time that depends on the degree of collateral blood flow. Recent clinical stroke trials with stent retrievers have demonstrated a benefit on neurologic outcome by endovascular thrombectomy in patients who present with a small volume of severe ischemia that has not yet enlarged[1–5] and who are thought to have good collateral blood flow.[6] However, many of these patients do not regain full neurologic recovery, and the probability of poor outcome increases progressively with the delay in reperfusion.[7,8] These findings have sparked renewed interest in discovering adjunct therapies that (1) can better maintain oxygen delivery through the collateral circulation before reperfusion is established and (2) provide neuroprotection after reperfusion is established.

In the above clinical trials some patients with apparently good collateral circulation benefited even when thrombectomy was delayed 6 to 8 hours.[5,8] Increasing O_2 delivery to the ischemic border region before clot lysis or removal would be expected to limit infarct growth and enhance the efficacy of clot lysis and removal. Strategies for increasing O_2 delivery include promoting dilation in the collateral arterial network, decreasing blood viscosity, increasing arterial O_2 content, and enhancing O_2 unloading in the microcirculation. One strategy to enhance O_2 unloading involves transfusion of cell-free hemoglobin (Hb) to facilitate O_2 diffusion from the red blood cell to the endothelium in capillaries with residual red blood cell perfusion. A plasma-based O_2 carrier also could deliver O_2 to capillaries with poor red blood cell perfusion but with persistent plasma perfusion. However, a Hb-based O_2 carrier with cross-linked tetramers failed in a clinical stroke trial,[9]

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Clinical Perspective

What Is New?

- Transfusion of polynitroxylated PEGylated cell-free hemoglobin, which possesses superoxide dismutase and catalase-mimetic activity, promoted cerebral vasodilation in the ischemic border region during experimental middle cerebral artery occlusion and resulted in decreased infarct volume; delaying transfusion until 2 hours of ischemia plus 2 hours of reperfusion also reduced infarct volume, thereby indicating a relevant therapeutic window.

What Are the Clinical Implications?

- Transfusion of this cell-free hemoglobin solution soon after the onset of ischemic stroke may help to sustain collateral blood flow until reperfusion occurs with thrombolytic or mechanical therapy, whereas delayed transfusion may still be of benefit by ameliorating reperfusion injury.

possibly because of nitric oxide scavenging and endothelin release.[10,11] Polymerization of Hb produces large molecules that do not normally extravasate or cause peripheral vasoconstriction.[12] Exchange transfusion of a large volume of 1 such polymer, 0-link polymer of bovine Hb (ZL-HbBv), after the onset of experimental MCA occlusion (MCAO) was more effective than exchange transfusion of cross-linked tetrameric Hb in reducing infarct volume.[13] However, the initial dilation of pial arteries in the ischemic border region gradually declined over a 2-hour period, and transfusion of the polymerized Hb did not reverse this loss of dilation.[14] This loss of pial arteriole dilation likely reflects a diminution of collateral blood flow. Therefore, we sought a modified Hb that would not only carry O_2 into the ischemic region but would also maintain pial arterioles in a dilated state and thus permit smaller volumes of Hb-based O_2 carrier transfusion.

Addition of polyethylene glycol (PEG) to Hb represents an alternative strategy to polymerization for increasing the molecular radius and reducing extravasation and consequent nitric oxide scavenging.[15,16] PEGylation also reduces antigenicity and increases oncotic pressure. Moreover, nitroxides act as superoxide dismutase (SOD) mimetics,[17] and addition of nitroxyl moieties on the Hb molecule can decrease an inflammatory response induced by endothelial oxidative stress.[18,19] Furthermore, the nitroxyl group can be reduced to a hydroxylamine form that then exerts peroxidase activity in the presence of ferric heme and thereby limit ferryl heme formation and consequent heme dissociation from Hb by H_2O_2.[20] This peroxidase activity is sufficient to inhibit H_2O_2-induced caspase activation. These characteristics of polynitroxylated PEGylated Hb (PNPH) may permit rescue of ischemic tissue by infusion of relatively small volumes. This approach would be simpler to implement clinically than using isovolumetric exchange transfusion of large volumes of earlier generations of Hb-based O_2 carriers.[13] Furthermore, the SOD-mimetic and peroxidase activity could provide benefit during reperfusion from MCAO. Therefore, PNPH may represent a viable adjunct therapy for endovascular treatment by stabilizing collateral blood flow and by mitigating reperfusion injury.

Here, we tested the a priori hypotheses that transfusion of 10 mL/kg of a 4% Hb solution of PNPH (1) reduces infarct volume when infused at 20 minutes after MCAO compared with no transfusion or transfusion of PEGylated albumin (PEG-albumin) or ZL-HbBv; (2) does not alter the diameter of pial arteries in nonischemic brain; (3) helps to maintain pial arteries in the distal MCA region in a dilated state when the duration of MCAO is prolonged to 2 hours; (4) increases perfusion in the ischemic border region when infusion is delayed until 90 minutes of MCAO; and (5) reduces infarct volume when infusion is delayed until 4, 6, and 8 hours after the onset of a 2-hour period of MCAO.

Methods

Infusion Solutions

PNPH was derived from purified bovine Hb that was conjugated with 5000-molecular-weight residues of PEG and equilibrated with carbon monoxide (CO) to prevent autoxidation to metHb. The PEGylated COHb was reacted with 4-(2-bromoactamido)-2,2,6,6-tetramethyl-1-piperidinyloxy to form covalently bound nitroxide moieties on the Hb molecule as described.[21] The polynitroxylation does not change the hemoglobin affinity for O_2 (P_{O_2} of ≈11 mm Hg at 50% saturation, 7.4 pH, 37°C). The solution was stored at a 4% Hb concentration at 4°C. Because PEGylation increases oncotic pressure, a 4% solution of PEGylated albumin was used as a control. Comparisons were also made with infusion of a 6% solution of ZL-HbBv in which nonpolymerized Hb was removed and very large polymers remained.[12,13] Because the large polymers have a relatively low oncotic pressure,[12] a higher concentration of 6% Hb was used in this solution compared with the 4% Hb in the PNPH solution.

Transient MCAO

Procedures on male Wistar rats (250-325 g; 8-12 weeks of age; Harlan Laboratories, Frederick, MD) were similar to those previously described[22] and were approved by the Johns Hopkins University Animal Care and Use Committee and conformed to the National Institutes of Health guidelines for the use of animals. Rats were anesthetized with 2% isoflurane in enriched O_2, rectal temperature was maintained at ≈37°C, and a femoral artery and vein were cannulated. Through an incision in the

scalp, the skull over the lateral parietal cortex was thinned with a drill, and a laser-Doppler flow (LDF) probe was secured against the translucent bone for monitoring perfusion in the core of the MCA territory. Through an incision in the neck, the right common carotid artery was occluded, the occipital artery was coagulated, the pterygopalatine artery was ligated, and a 4-0 monofilament nylon suture with a rounded tip was advanced into the internal carotid artery until a stable reduction in LDF was achieved. Reperfusion was produced by withdrawal of the monofilament after 2 hours of occlusion and was confirmed by LDF monitoring. Mean arterial blood pressure (MABP), LDF, temperature, and arterial blood gases were monitored during MCA occlusion and early reperfusion. Infarct volume measurements were made at 1 or 3 days of reperfusion.

The intravenous transfusions of 10 mL/kg were performed over a 6-minute duration. Rats with an initial LDF above 40% of the preischemic baseline were excluded before transfusion. Because transfusion of PNPH resulted in plasma samples with a red color, the person performing the procedures was not blinded to treatment. Within each experiment, treatment groups were performed concurrently, although they were not strictly randomized. Surgeries on rats receiving PNPH at 6 or 8 hours after MCAO were started in the morning, and surgeries on rats without a transfusion or receiving PNPH at 4 hours after MCAO were started in the afternoon.

Infarct Volume

Brains were sectioned into 7 slabs and stained with the vital dye triphenyltetrazolium chloride. The vital and nonvital stained areas on each side of each slab were measured by an observer blinded to treatment for the calculation of the percentage of infarcted volume. Adjustments for swelling were made by multiplying the infarct volume by the ratio of the contralateral-to-ipsilateral volume of the entire structure. Based on the SD of previous experiments,[22] a sample size of 10 for single comparisons and 14 for 3 comparisons was estimated to provide 80% power for detecting differences in infarct volume of 21% of hemisphere volume at the 0.05 significance level. Mortality was <25% in each group, and all survivors were included in the analysis.

Pial Arteriole Diameter

As previously described,[14] we performed a 3- to 4-mm craniotomy lateral to the sagittal suture and caudal to the coronal suture in mechanically ventilated rats. A plastic ring with side ports for measuring fluid pressure and temperature was cemented to the skull and filled with artificial cerebrospinal fluid. The dura was cut and gently retracted, and the ring was sealed with a glass coverslip for intravital microscopy. We averaged the percentage change in diameter from baseline of pial arterioles at 3 to 6 sites to obtain a single value per rat for statistical analysis.

Perfusion in the Ischemic Border Region

In addition to measuring LDF in the ischemic core in all rats at 10 mm lateral from the bregma, we measured LDF at a second site 4 mm caudal and 3 mm lateral from the bregma in a subset of mechanically ventilated rats. This site has a smaller reduction in LDF during MCAO and was assumed to represent the ischemic border region.[14,22]

Statistical Analysis

Because some of the infarct volume distributions did not pass the normality test, nonparametric tests were used to test effects of treatments on infarct volume. In the first experiment, infarct volumes in a control group with no transfusion (n=10) and groups transfused with PNPH (n=10), PEG-albumin (n=10), and ZL-HbBv (n=5) at 20 minutes of MCAO were compared with the Kruskal-Wallis test. If the differences in the median values were significant ($P<0.05$), individual groups were compared by the Dunn method for multiple comparisons. In a second experiment the effect of PNPH transfusion on pial artery diameter in rats without MCAO was analyzed by repeated-measures ANOVA (n=7). In a third experiment the percentage change in diameter after MCAO was compared between groups either with no transfusion (n=8) or with PNPH transfusion (n=7) by t test. In a fourth experiment rats were transfused with PNPH at 90 minutes of MCAO, and LDF values in the ischemic core and in the ischemic border region were compared before and 30 minutes after transfusion by paired t test (n=8). In a fifth experiment that investigated the effect of PNPH transfusion during reperfusion, comparisons of infarct volumes between a nontransfused control group (n=15) and groups transfused with PNPH at 4 (n=14), 6 (n=14), or 8 (n=14) hours after MCAO were analyzed by the Kruskal-Wallis test; comparisons with the control group were made with the Dunn test. Physiologic data were analyzed among groups at specific time points by 1-way ANOVA, and comparisons with the control group were made with the Dunnett test. $P<0.05$ was considered significant in all tests. Unless otherwise noted, data are expressed as means±SE.

Results

Blood Analysis, Hemodynamics, and Infarct Volume Following PNPH Transfusion During Transient MCAO

In the first experiment rats were transfused at 20 minutes of MCAO with 10 mL/kg PNPH without an equivalent withdrawal

of blood (topload transfusion). The transfusion increased plasma [Hb] to 0.4±0.1 g/dL at 60 minutes of MCAO, and [Hb] remained at this level at 30 minutes of reperfusion. Total blood [Hb] was not significantly increased (12.2±0.3 to 11.9±0.6 g/dL after transfusion), due, in part, to the relatively small increase in plasma [Hb] and possibly to the 0.7 mL blood sample drawn for whole blood and plasma analysis. Because PNPH was synthesized and stored in the carboxy state, it released CO after transfusion. Whole-blood COHb increased from 0.6±0.1% to 1.7±0.1% at 60 minutes of MCAO and recovered to 0.7±0.2% by 30 minutes of reperfusion. Whole-blood metHb increased from 0.8±0.1% to 1.4±0.1% at 60 minutes of MCAO and remained at this level at 30 minutes of reperfusion. Arterial pH (7.40±0.01), P_{CO_2} (45±2 mm Hg), and P_{O_2} (118±4 mm Hg) remained in the normal physiologic range.

Prior to transfusion MCAO produced an immediate decrease in LDF in the lateral cortex (ischemic core region) to ≈30% of baseline. The transfusion of PNPH, PEG-albumin, or ZL-HbBv did not produce significant arterial hypertension or improve LDF in the ischemic core (Figure 1A and 1B). Core LDF after transfusion of PNPH was similar to that in the other 3 groups throughout the 2 hours of MCAO. Figure 1C shows the individual infarct volume values, measured at 1 day after MCAO, along with the box-whisker plots of the medians and interquartile and 5% to 95% ranges for each group. The Kruskal-Wallis test indicated an overall effect of treatment group on infarct volume in cerebral cortex ($P<0.001$) and in striatum ($P<0.001$). Individual comparisons indicated that infarct volume in these regions was significantly smaller in the PNPH-transfused groups compared with the control group with no transfusion and to the groups transfused with PEG-albumin and ZL-HbBv; the latter 3 groups did not differ from each other.

Effect of PNPH Transfusion on Arterial Diameter Without MCAO

We examined whether transfusion of 10 mL/kg PNPH had any effect on pial arteriole diameter in a group of rats without ischemia. Repeated-measures ANOVA indicated no significant effect ($P=0.45$) of PNPH transfusion on pial arteriole diameter over the 2-hour observation period (Figure 2B). MABP remained close to baseline from 15 to 120 minutes after transfusion (Figure 2A). Compared with baseline, arterial pH (7.38±0.01 to 7.42±0.01), P_{CO_2} (43±1 to 41±1 mm Hg),

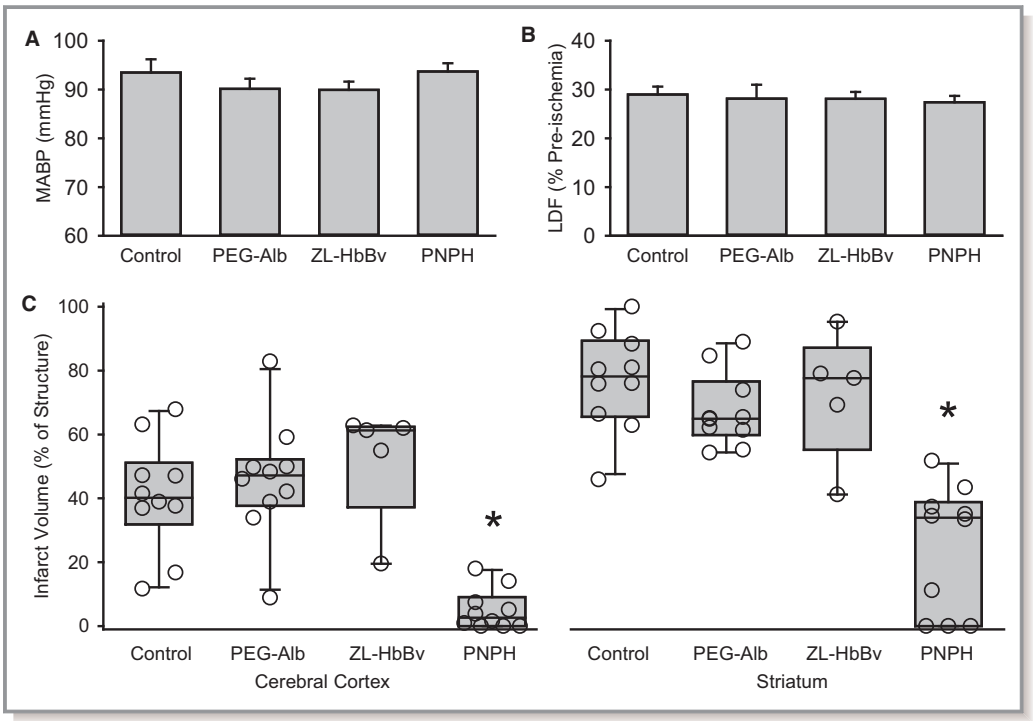

Figure 1. Mean±SE of mean arterial blood pressure (A, MABP) and lateral parietal laser-Doppler flow (B, LDF) in ischemic core averaged over 2 hours of middle cerebral artery occlusion (MCAO) in a control group with no transfusion (n=10) and groups transfused with PEG-albumin (PEG-Alb; n=10), 0-link polymerized bovine hemoglobin (ZL-HbBv; n=5), and polynitroxylated PEGylated hemoglobin (PNPH; n=10) at 20 minutes of MCAO. C, Individual values (open circles) and box-whisker plots (5th, 25th, 50th, 75th, and 95th percentiles) of infarct volume in cerebral cortex and striatum at 1 day of recovery. *$P<0.05$ from control group.

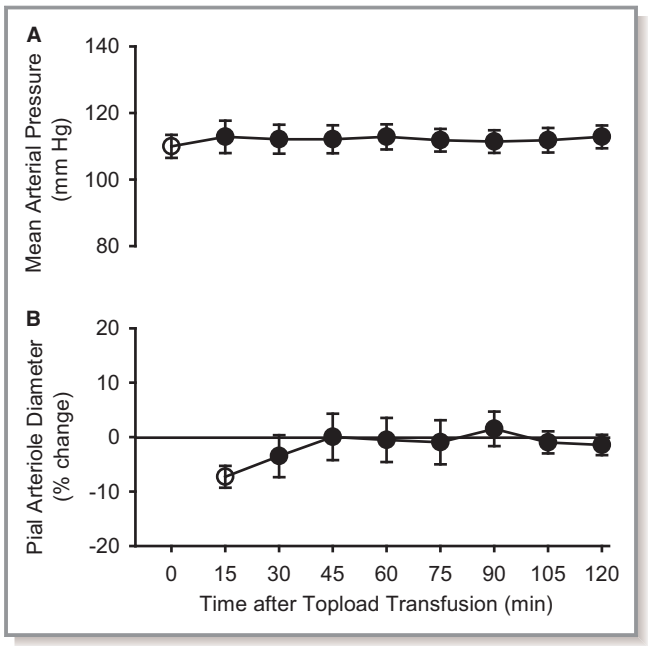

Figure 2. Mean±SE of mean arterial blood pressure (A) and percentage change in pial arteriole diameter (B) after transfusion of polynitroxylated PEGylated hemoglobin in 7 rats without induction of ischemia.

and [Hb] (13.1±0.4 to 12.8±0.4 g/dL) were not substantially changed 2 hours after transfusion. Arterial P_{O_2} was in the 100 to 150 mm Hg range. Thus, the physiologic status of the rats remained stable after transfusion.

Effect of PNPH Transfusion on Arterial Diameter During MCAO

Next, we examined the effect of PNPH transfusion at 20 minutes after the onset of MCAO on pial arteriole diameter in the distal MCA territory. In control rats with no transfusion, pial arteriole diameter in the distal MCA territory initially increased by 36±2% of the preischemic value (Figure 3E). However, dilation gradually decreased to 5±4% by 2 hours of MCAO. In the group transfused with 10 mL/kg PNPH, pial arteriole diameter increased by 31±3% before the transfusion. At 2 hours of MCAO, diameter remained 21±4% above the preischemic value in the PNPH-transfused-group. This increase in diameter was significantly greater than that seen in the group with no transfusion. In contrast to the observed changes in diameter, repeated-measures ANOVA and the Dunnett test did not reveal a significant change in arterial [Hb], hematocrit, arterial P_{CO_2}, or MABP after MCAO in either group (Figure 3A through 3D). Thus, the different time-dependent changes in diameter were not attributable to changes in key physiological regulatory factors of vascular control.

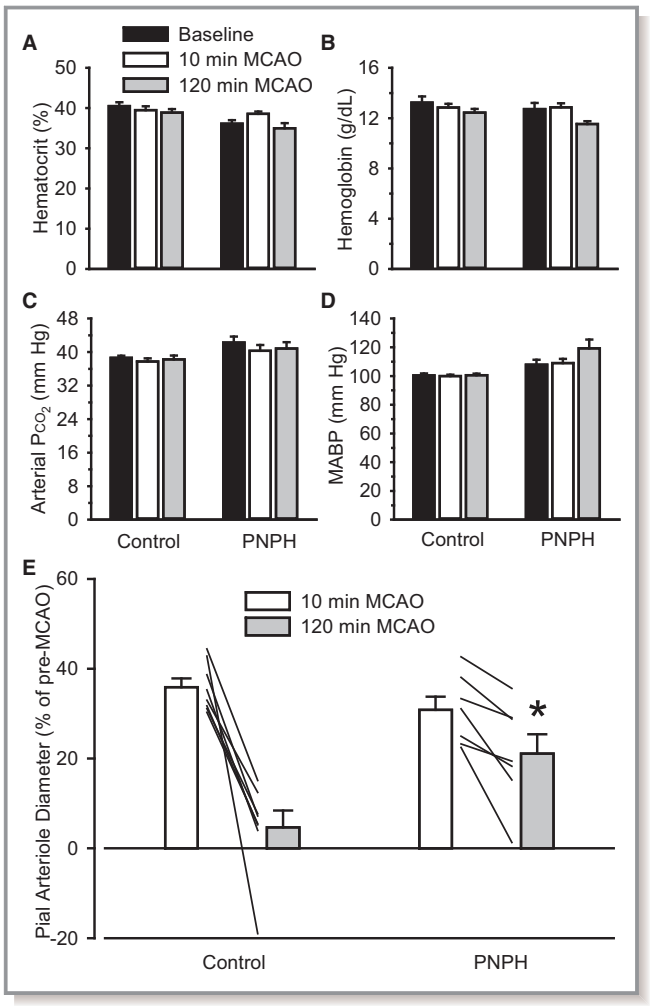

Figure 3. Mean±SE of arterial hemoglobin concentration (A), arterial hematocrit (B), arterial partial pressure of CO_2 (P_{CO_2}; C), and mean arterial blood pressure (MABP; D) at baseline and at 10 and 120 minutes of middle cerebral artery occlusion (MCAO) in a control group undergoing no transfusion (n=8) and in a group with polynitroxylated PEGylated hemoglobin (PNPH) transfusion (n=7) at 20 minutes of MCAO. Corresponding percent changes in pial arteriole diameter from preischemic baseline are shown for group mean±SE and paired individual changes between 10 and 120 minutes of MCAO (E). *P<0.05 from control group at corresponding time.

Penumbral Blood Flow With Delayed PNPH Transfusion

We determined whether topload transfusion of PNPH increases perfusion in the ischemic border region when transfusion was delayed by 90 minutes. LDF in the border region before transfusion was 48±6% of the preischemic baseline (Figure 4A). At 30 minutes after transfusion (2 hours of MCAO), it increased significantly to 67±8% (P<0.005, paired t test). In the ischemic core, LDF was not significantly improved (P=0.052). Cerebral O_2 transport was calculated as the product of arterial O_2 content and LDF. Arterial O_2 content was 15.5±0.3 mL O_2/dL before the transfusion and

15.9±0.4 mL O_2/dL after the transfusion (no significant difference). However, cerebral O_2 transport was significantly increased after transfusion both in the border region (P=0.008) and in the core region (P=0.038, Figure 4B).

Infarct Volume After Delayed PNPH Transfusion

Transfusion of polynitroxylated albumin at 2 hours of reperfusion has been reported to reduce infarct volume in the rat.[23] We tested whether transfusion of PNPH at 4 hours after MCAO, which corresponded to 2 hours of reperfusion, was effective in reducing infarct volume and if a protective effect persisted with a 6- and 8-hour delay from the onset of MCAO. Arterial [Hb] was slightly higher in the group later transfused with PNPH at 4 hours, and rectal temperature was slightly lower in the control group than in the other groups (Figure 5). Arterial P_{CO_2}, arterial pH, MABP, and LDF in the ischemic core in the groups later undergoing transfusion were not significantly different from control-group values during MCAO or at 30 minutes of reperfusion (Figure 5). Thus, the physiological insult was comparable among groups before PNPH transfusion.

Infarct volume was measured 3 days after MCAO and compared among the 4 groups with the Kruskal-Wallis test. Significant differences were detected among groups (P=0.041). Post-hoc analysis with the Dunn test indicated that transfusion with PNPH at 4 hours after MCAO significantly reduced infarct volume (Figure 6). With transfusion at 6 or 8 hours, infarct volume was more variable than those with a 4-hour treatment delay; some of the rats had small infarct volumes, but others showed larger infarcts comparable to the control group. Thus, the reduction in infarct volume was no longer statistically significant with 6- or 8-hour delays.

Discussion

We demonstrated several new findings in this study. First, topload transfusion with 10 mL/kg of PNPH in rats at 20 minutes after the onset of MCAO markedly reduced infarct volume. Second, transfusion with PNPH had no major effect on pial arterioles in nonischemic brain. Third, pial arterioles in the distal MCA region were maintained in a vasodilated state at 2 hours of MCAO when PNPH was transfused at 20 minutes of MCAO. Fourth, delaying the transfusion of PNPH until 90 minutes of MCAO improved LDF in the ischemic border region. Fifth, delaying the transfusion until 4 hours after the onset of MCAO (2 hours of reperfusion) reduced infarct volume. Thus, PNPH has a significant therapeutic window of opportunity and can be effective when transfused during transient MCAO as well as after reperfusion.

These beneficial effects of a topload transfusion of PNPH were achieved with a relatively small transfusion volume of 10 mL/kg of a 4% Hb solution. In contrast, transfusion of a ZL-HbBv required exchange transfusion with ≈40 mL/kg of a 6% Hb solution to significantly reduce infarct volume; exchange transfusion with 40 mL/kg of a 3% solution[13] or topload transfusion with 10 mL/kg of a 6% solution[22] were less effective. Likewise, exchange or topload transfusion of a 5% solution of non-PEGylated human serum albumin was ineffective in reducing infarct volume.[13,22] In the present study, we confirmed that small volume transfusion of PEG-albumin or ZL-HbBv did not reduce infarct volume. These

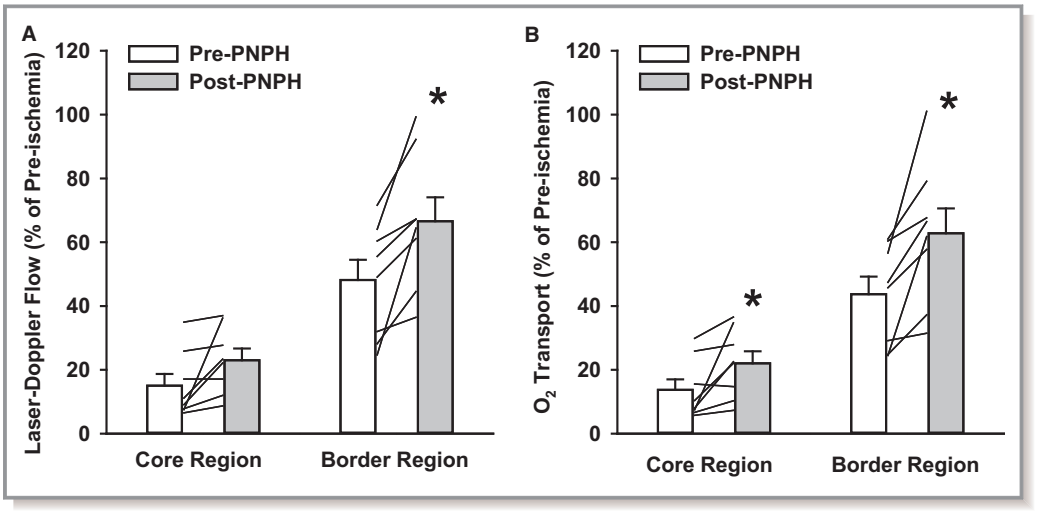

Figure 4. A, Laser-Doppler flow measured in ischemic core region and border region of cerebral cortex before the start of polynitroxylated PEGylated hemoglobin (PNPH) topload transfusion at 90 minutes of middle cerebral artery occlusion and 30 minutes later. B, Corresponding changes in cerebral O_2 transport calculated from the product of arterial O_2 content and laser-Doppler flow (expressed as a percentage of preischemic baseline). Paired individual changes in blood flow and O_2 transport before and after transfusion are shown for 8 rats together with the mean±SE. *P<0.05 from pretransfusion value.

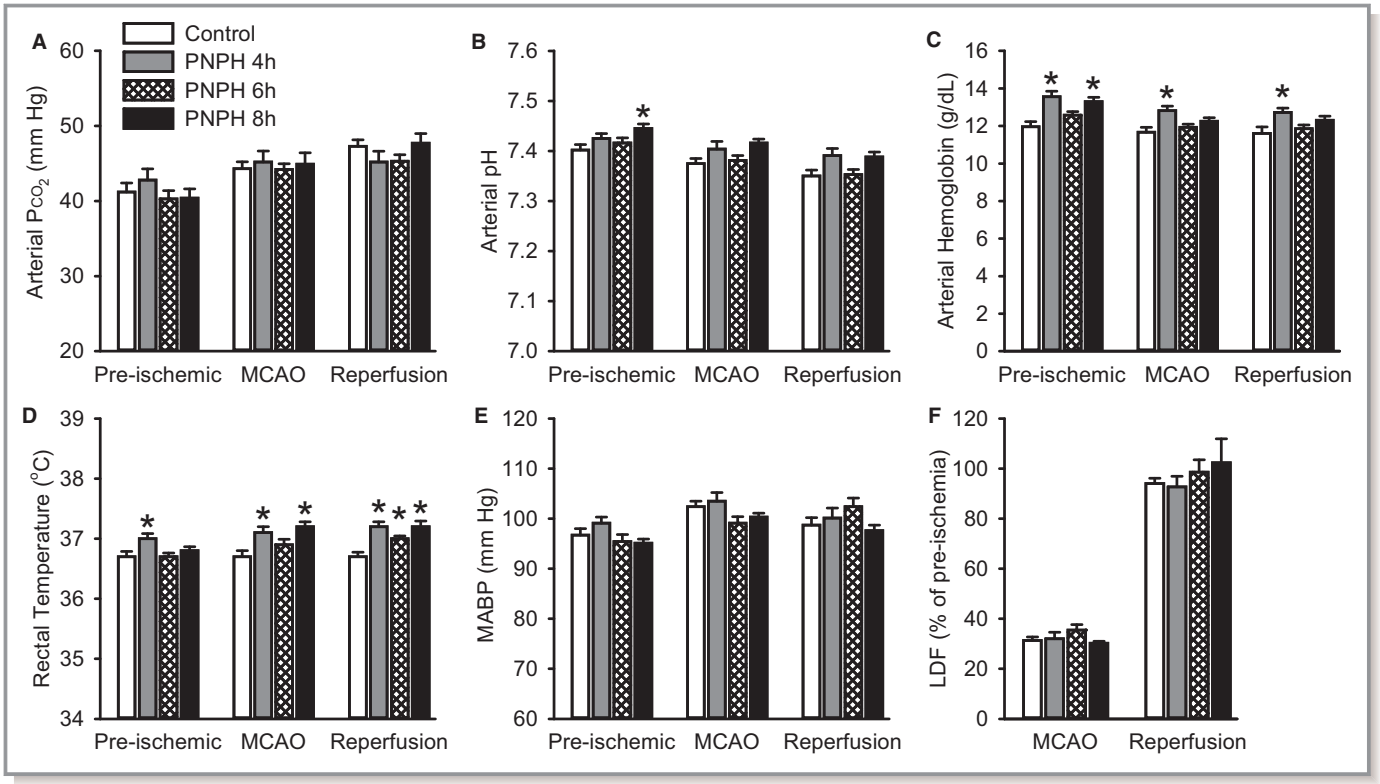

Figure 5. Mean±SE of arterial partial pressure of CO_2 (P_{CO_2}; A), arterial pH (B), arterial hemoglobin concentration (C), rectal temperature (D), and mean arterial blood pressure (MABP; E) preischemia, during middle cerebral artery occlusion (MCAO), and during reperfusion in a control group with no transfusion (n=15) and in groups that later received a polynitroxylated PEGylated hemoglobin (PNPH) topload transfusion at 4, 6, or 8 hours after the onset of MCAO (n=14 each) and survived for 3 days for infarct volume analysis. *P<0.05 from the no transfusion group. The percentage change in laser-Doppler flow (LDF) over lateral parietal cortex was not different among the 4 groups during MCAO and reperfusion (F).

comparisons suggest that PNPH exerts a protective effects in a stroke model beyond simply its effects on oncotic pressure and oxygen-carrying capacity of blood. The plasma [Hb] of only 0.4 g/dL achieved with the 10 mL/kg transfusion of PNPH had negligible effects on whole blood [Hb] and arterial O_2 content. Thus, changes in oxygen-carrying capacity are too small to account for the large decrease in infarct volume observed after PNPH transfusion.

One mechanism by which PNPH could decrease infarct volume is by promoting vasodilation. We previously reported progressive loss of pial arteriole dilation in the MCA border region as the duration of MCAO was extended to 2 hours.[14] In the present study, we found that topload transfusion with 10 mL/kg of PNPH resulted in significantly greater vasodilation at 2 hours compared with the control group. In contrast, exchange transfusion with ZL-HbBv did not prevent the loss of vasodilation,[14] thereby indicating that improved vasodilation was not a characteristic of all cell-free Hb.

The reduction in infarct volume seen with PNPH transfusion at 20 minutes of MCAO was not associated with significant changes in arterial blood gases, large increases in MABP, or improvements in LDF in the lateral parietal cortex, which is presumed to be in the cortical ischemic core region of the MCA distribution. However, in these survival experiments we did not measure LDF in the ischemic border region subserved by collateral vessels and where sustained dilation of pial arterioles likely reflects sustained collateral blood flow. Nevertheless, in another cohort we did find that LDF in the MCA border region increased to 67% of the preischemic baseline level when transfusion was delayed until 90 minutes after MCAO (Figure 4A). This increase in perfusion resulted in a parallel increase in O_2 transport into the border region (Figure 4B). Because of the brain's ability to substantially increase O_2 extraction, levels of blood flow of 67% of baseline may have been sufficient to sustain viability in the ischemic border region. Therefore, improved perfusion in the cortical border region likely contributes to the decrease in infarct volume in cerebral cortex when the transfusion was performed during the ischemic period.

In a previous study,[22] preserved pial arteriole dilation in the MCA border region was also attained with topload infusion of PEG-COHb, whereas infusion of PEG-Hb without bound CO or with crosslinked Hb that was not PEGylated was less effective in preserving vasodilation. More recently, increased collateral blood flow has been reported after MCAO in spontaneously hypertensive rats,[24] which are known to have leptomeningeal

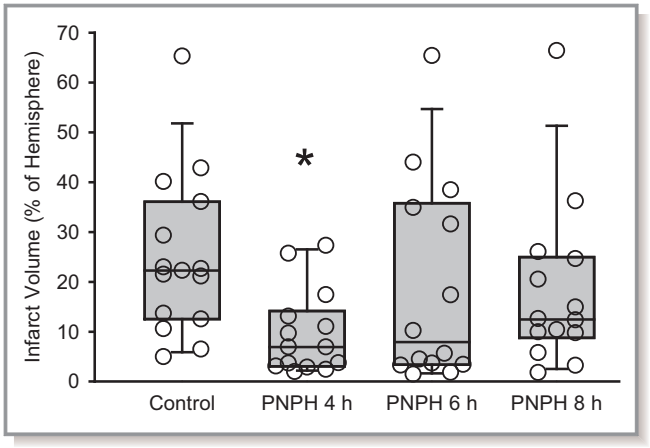

Figure 6. Individual values (open circles) and box-whisker plots (5th, 25th, 50th, 75th, and 95th percentiles) of infarct volume in cerebral hemisphere at 3 days of recovery in a control group with no transfusion (n=15) and in groups receiving a polynitroxylated PEGylated hemoglobin (PNPH) topload transfusion at 4, 6, or 8 hours after the onset of middle cerebral artery occlusion (n=14 each). **P*<0.05 from control group.

arterioles with high tone.[25] Because PNPH is PEG-COHb with nitroxide adducts, our results do not distinguish whether the benefit of PNPH on vasodilation is attributable to release of CO or to SOD-mimetic and peroxidase activity. In either case, PNPH may help stabilize collateral blood flow.

The actions of some HBOCs to produce peripheral vasoconstriction and increase MABP are related to nitric oxide scavenging.[11,26] In our study, the change in MABP was minimal. In the absence of ischemia, transfusion of PNPH did not produce significant constriction of pial arterioles. Hence, PNPH does not appear to scavenge sufficient nitric oxide to constrict pial arterioles or to increase MABP.

Interestingly, PNPH transfusion at 20 minutes of MCAO reduced infarct volume in striatum. Because striatum is considered an end-artery region, the substantial reduction in striatal infarct volume suggests that PNPH may also act by mechanisms independent of increasing collateral blood flow. We previously reported that PEGylated SOD administration can reduce striatal injury from MCAO.[27] Thus, PNPH may protect the striatum by virtue of its SOD-mimetic activity as well as possible prosurvival effects of released CO.[28]

The SOD-mimetic activity of PNPH may also protect against reperfusion injury. In support of this possibility, polynitroxylated albumin infusion during MCAO[29] or as late as 2 hours of reperfusion after 90-minute MCAO[23] has been reported to reduce infarct volume. Thus, we postulated that PNPH would also be beneficial when transfused after reperfusion. We found a significant reduction in infarct volume in rats transfused at 4 hours after MCAO (2 hours of reperfusion). Even in groups transfused at 6 and 8 hours after MCAO, some of the rats had relatively small infarcts, although these groups as a whole were not statistically different from the control group. Therefore, PNPH has a considerable therapeutic time window for the situation in which reperfusion can be established.

Some caution is required in interpreting the results of the filament model because it does not fully simulate thrombus-endothelial interactions, effects of tissue plasminogen activator administration on the blood-brain barrier, or variability in effective recanalization. The filament may also produce some damage of endothelium in the internal carotid artery. Nevertheless, the rapid reperfusion attained with withdrawal of the filament does elicit a reperfusion injury that is likely to be analogous to that occurring in patients with endovascular thrombectomy and successful reperfusion.

Some capillaries may have poor reflow during reperfusion after prolonged ischemia.[30] Because the flow of plasma may persist in narrowed capillaries, PNPH may gain access to capillaries with poor red blood cell flux and thereby improve oxygen delivery. However, limited oxygen delivery in postischemic tissue might fuel the generation of reactive oxygen species. Thus, adding SOD-mimetic activity to an oxygen carrier may augment the neuroprotective efficacy of the plasma-based Hb.

One concern with increasing Hb in the plasma is that the Hb may extravasate and exert toxic effects on neurons, particularly if PNPH transfusion is delayed during reperfusion after the barrier has been disrupted. However, adding PNPH to the media of cultured neurons has been found not to augment neuronal cell death; rather, neurons were protected from native Hb and glutamate excitotoxicity.[21] Moreover, PNPH transfusion was found to be neuroprotective in a combined model of hemorrhagic shock and traumatic brain injury.[21,31] Therefore, PNPH appears to be safe to use during the first few hours of reperfusion after transient MCAO.

This study does have some limitations in that PNPH was not tested in female animals, aged animals, or in a model of permanent MCAO. Moreover, optimal dosing regimens and long-term behavioral outcomes were not evaluated. Nevertheless, the considerable reduction in infarct volume with a relatively small, single infusion volume of PNPH indicates that this agent holds promise and warrants further investigation in preclinical stroke models. Moreover, the ability of PNPH to promote vasodilation when infused before reperfusion and to sustain infarct reduction when infused after reperfusion suggests pleiotrophic actions that would be an attractive feature for adjunct therapy with endovascular thrombectomy.

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Disclosures

Hsia holds shares in SynZyme Technologies, which holds the license for polynitroxylated PEGylated hemoglobin. There are no other relevant financial disclosures or conflict of interests by the remaining authors.

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