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Focal Cerebral Ischemia in Rats

Effect of Hypervolemic Hemodilution with Diaspirin Cross-linked Hemoglobin Versus Albumin on Brain Injury and Edema

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Background: Hemodilution has had limited success as a treatment of cerebral ischemia. When using a non-oxygen-binding fluid, the therapeutic efficacy of hemodilution-induced increases in blood flow are offset by concomitant decreases in oxygen content.

Methods: The effect of hemodilution, with diaspirin cross-linked hemoglobin (DCLHb), on brain injury and edema was assessed during middle cerebral artery occlusion (180 min) and reperfusion (120 min) in rats (blood volume increased by $\approx 30\%$ and $n = 10$ for each group): (1) 44/B: 8.0 ml of donor blood was given; (2) 30/albumin: hematocrit was decreased to 30% with 10% albumin; (3) 30/DCLHb: hematocrit was decreased to 30% with 10% DCLHb; or (4) 9/DCLHb: hematocrit was decreased to 9% with DCLHb. Infarct size was analyzed with 2,3,5-triphenyltetrazolium chloride, and edema by microgravimetry.

Results: Brain injury (percent of the hemispheric area ipsilateral to ischemia, mean \pm SD) was greater in the 44/B group (44 ± 4) versus the 30/albumin group (37 ± 3). In addition, brain injury was greater in the 44/B and 30/albumin groups versus the 30/DCLHb group (27 ± 4); which was in turn greater than the 9/DCLHb group (18 ± 3). Specific gravity was greater (less brain water) in all hemodiluted groups versus the 44/B group.

Conclusions: These results support a hypothesis that hemodilution decreases focal cerebral ischemic injury, and when an oxygen-binding fluid is used, there is a dose-dependent effect of hemodilution on ischemia. In addition, these results

suggest that hemodilution, as achieved with DCLHb, was more effective in reducing ischemic brain damage than was the same degree of hemodilution as achieved with albumin. (Key words: Blood: hemodilution; hemoglobin. Brain: focal cerebral ischemia.)

DURING surgical procedures in which the patient is at risk for cerebral ischemia, hemodilution may ameliorate brain injury.¹ The rationale for hemodilution therapy is based on evidence that correlates a decrease in viscosity (hematocrit the major determinant) with an increase in cerebral blood flow (CBF).²⁻⁷

The rationale for hemodilution is sound, but the results of therapeutic trials have been inconsistent.⁶⁻¹³ Although many reasons may account for this diverse response (*e.g.*, magnitude of hematocrit reduction, treatment delay, side effects^{14,15}), one explanation with merit is a decrease in oxygen content and inherent limitation in oxygen transport when non-oxygen-binding fluids are employed. Thus, an increase in oxygen transport induced by augmenting CBF may be negated by a concurrent decrease in oxygen-carrying capacity. As the steepest portion of the viscosity-hematocrit curve is at hematocrits greater than 30%,² hemodilution therapy has been restricted to modest reductions in hematocrit.² It is proposed that, if hematocrit reductions are limited to this range, the benefit from an increase in CBF will exceed the disadvantage from reduced oxygen content, and oxygen delivery will increase. Accordingly, the magnitude of therapy in previous trials has been limited.

In a previous study, we observed that hemodilution with diaspirin cross-linked hemoglobin (DCLHb) decreased ischemia during middle cerebral artery occlusion (MCAo) in rats.¹⁶ This augmentation of CBF was dose-dependent with the optimal effect observed at a hematocrit of 9% (total hemoglobin 8.3 g/dl). In the present study, we compared the effect of hemodilution

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with DCLHb *versus* albumin on brain injury and edema during temporary MCAo in rats.

Materials and Methods

The DCLHb solution, obtained from Baxter Healthcare (Deerfield, IL), was made according to Chatterjee *et al.*¹⁷ Human erythrocytes were lysed, the hemolysate was centrifuged, and stroma lipids were removed. After ultrafiltration, the diaspirin compound bis(3,5-dibromosalicyl) fumarate was used to cross-link molecular hemoglobin at the α chain. Viral contamination was eliminated and protein purification achieved by heat pasteurization.^{18,19} The final DCLHb solution had a hemoglobin concentration of 10.2 g/dL (table 1). The solution was stored at -70° C. When needed for the present study, DCLHb was thawed to 5° C and passively warmed to room temperature. Oxygen transport of DCLHb is similar to whole blood¹⁷ with a slight right-shift in the oxygen dissociation curve.²⁰ The α - α cross-linking with bis(3,5-dibromosalicyl) fumarate prolongs the intravascular half-life (≈ 24 h²¹). The viscosity of DCLHb (1.3 centistokes) is comparable to serum albumin²² and considerably less than whole blood (>4.0 centistokes).²³

The protocol was approved by the Animal Research Committee of Loma Linda University in accordance with the National Institutes of Health standards for laboratory animals (NIH pub. no. 85-23, 1985). Male spontaneously hypertensive rats (weight 350–400 g, age 16–20 weeks) were anesthetized with 1.2 MAC isoflurane (1.44% end-tidal²⁴), orotracheally intubated and ventilated with a Harvard Rodent Respirator (Boston, MA). The femoral vessels were cannulated for con-

tinuous blood pressure monitoring (Full Scale Transducer/TA 2000 Recorder, Gould, Cerritos, CA), blood sampling, and fluid administration. Cranial temperature was servo-controlled at 37° C with a heating blanket. Arterial blood (125 μ l) was collected at 30-min increments throughout the study and analyzed for pH, PaCO₂, PaO₂, glucose, and hematocrit (IL-1306 pH Blood Gas Analyzer, Instrumentation Laboratory, Lexington, MA; YSI Model 23-A Glucose Analyzer, Yellow Springs Instruments, Yellow Springs, OH; IEC MB Centrifuge Microhematocrit, DAMON/IEC Division, Needham Heights, MA). All physiologic data are reported as an average over the entire study period (MCAo and reperfusion).

Part A

Each rat was randomized to one of the following groups for which blood volume was increased by 8.0 ml ($\approx 30\%$). The hypervolemic-hemodilution regimen was initiated 90-min prior to MCAo and was maintained at steady state for at least 60-min before MCAo and throughout the study. For maintenance fluid, 0.9% NaCl was given at $4 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$:

44/B ($n = 10$): Blood volume was increased by giving 8.0 ml of donor blood (hematocrit not manipulated).

30/albumin ($n = 10$): Blood volume and hematocrit (30%) were manipulated by a 5.0-ml exchange transfusion with 10% human albumin solution (Travenol Laboratories, Glendale, CA) followed by an 8.0-ml infusion of 10% albumin. Hemodilution with albumin was expected to decrease mean arterial blood pressure (MABP)¹²; accordingly, during MCAo, phenylephrine (0.1%) was required to maintain MABP at control values.

30/DCLHb ($n = 10$): Blood volume and hematocrit (30%) were manipulated by a 5.0-ml exchange transfusion with 10% DCLHb (Baxter Healthcare, Deerfield, IL, lot 2905T008), followed by an additional 8.0-ml infusion of 10% DCLHb.

9/DCLHb ($n = 10$): Blood volume and hematocrit (9%) were manipulated by a 20.0-ml exchange transfusion with DCLHb, followed by an additional 8.0-ml infusion of DCLHb.

Each exchange transfusion was accomplished by simultaneously withdrawing and infusing the appropriate solution at a rate of 1.0 ml/min. When given as a bolus, hemoglobin substitutes increase MABP.²⁵ However, in this species, if DCLHb is given initially as an exchange

Table 1. Chemical Assay of 10% Diaspirin Cross-linked Hemoglobin Solution*

Hemoglobin content	10.2 g/dl
Methemoglobin	0.7 g/dl
p50 (37° C)	32.0 mmHg
Osmolality	290 mOsm/kg
Oncotic pressure	42.7 mmHg
Viscosity	1.3 centistokes
pH	7.50
Na ⁺	140 mEq/L
K ⁺	5.0 mEq/L
Ca ⁺⁺	2.2 mEq/L
Mg ⁺⁺	1.0 mEq/L
Cl ⁻	115 mEq/L
Lactate	30 mEq/L

* Prepared by Baxter Healthcare, Deerfield, IL (lot 2905T008).

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transfusion, normotension is maintained. Moreover, in the DCLHb groups, hemodilution did not decrease MABP and phenylephrine was not required.

Via a subtemporal craniectomy, MCAo was achieved with 10-0 monofilament nylon suture (proximal to the lenticulostriate branch and distal to the inferior cerebral vein) to achieve ischemia to both cortical and subcortical tissue.^{12,14} Occlusion was maintained for 180-min, after which the sutures were released and 120 min of reperfusion allowed. During the study, the craniectomy site was bathed in mock cerebral spinal fluid (126.5 mM NaCl, 27.5 mM NaHCO₃, 2.4 mM KCl, 0.5 mM K₂HPO₄, 1.1 mM CaCl₂, 0.85 mM MgCl₂, 0.5 mM Na₂SO₄, 5.9 mM glucose, pH 7.5, 37° C).

After MCAo and reperfusion, the brains were removed and sectioned in coronal planes 3.0 and 5.0 mm from the frontal pole (fig. 1). The middle segment was immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) at 37° C for 30 min. Each brain surface (3.0- and 5.0-mm coronal planes) was photographed with color slide film (Ektachrome, tungsten 160 ASA), and the area of brain injury (fig. 2) determined with a Drexel/DUMAS Image Processing System (Philadelphia, PA).²⁶ All image analysis was performed by an independent observer who was blinded to study protocol.

Tissue was obtained for microgravimetry from the 1.0–3.0- and 5.0–7.0-mm brain segments (figs. 1 and 3). Parallel specimens of cortex and basal ganglia were sampled from both hemispheres with a 2.0-mm biopsy

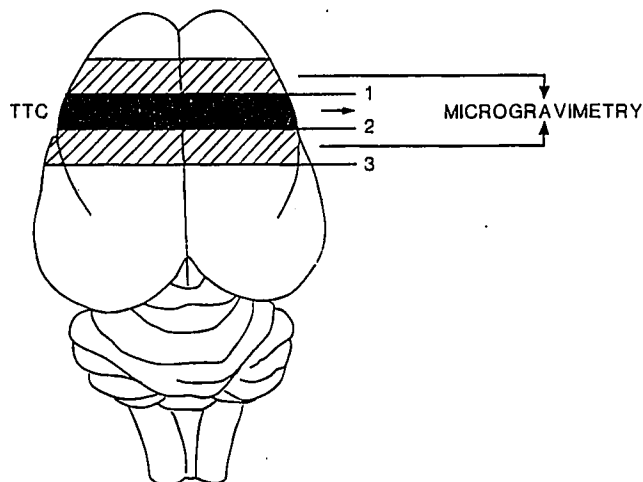


Fig. 1. Method of segmenting the brain for analysis of injury (solid) and edema (hatched). 1 = the coronal plane 3 mm from the frontal pole; 2 = the coronal plane 5 mm from the frontal pole; 3 = the coronal plane 7 mm from the frontal pole.

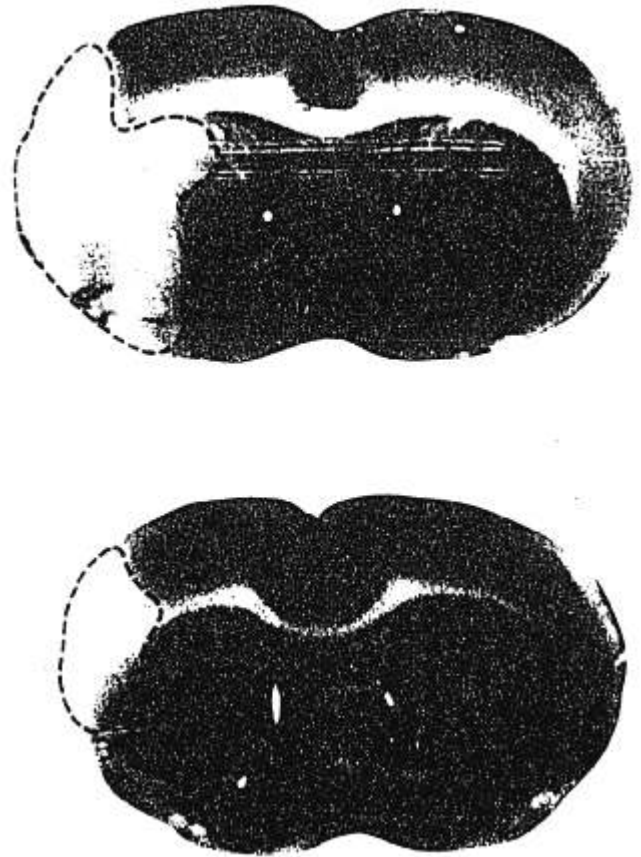


Fig. 2. 2,3,5-triphenyltetrazolium chloride stained brain section (3.0 mm from the frontal pole) in the 44/B and 9/DCLHb groups. The dark area corresponds to normal brain, and the pale area within the dashed line was defined as injured brain.

punch (Baker and Cummins, Miami, FL). Specific gravity was measured by placing the tissue specimens in a kerosene-bromobenzene density gradient. The linear regression equation for each gradient was determined and verified with potassium sulfate standards.²⁷

Part B

With the exception of a craniotomy, different spontaneously hypertensive rats were prepared identical to Part A ($n = 5$ for each group). With the use of an IL-282 Co-oximeter (Instrumentation Laboratory), and at the appropriate hemodilution endpoint, arterial blood was collected and analyzed for hematocrit, total hemoglobin, and oxygen content according to the method described by Dennis and Valeri.²⁸

The physiologic data were analyzed by an analysis of variance with Scheffe's test for multiple comparisons.

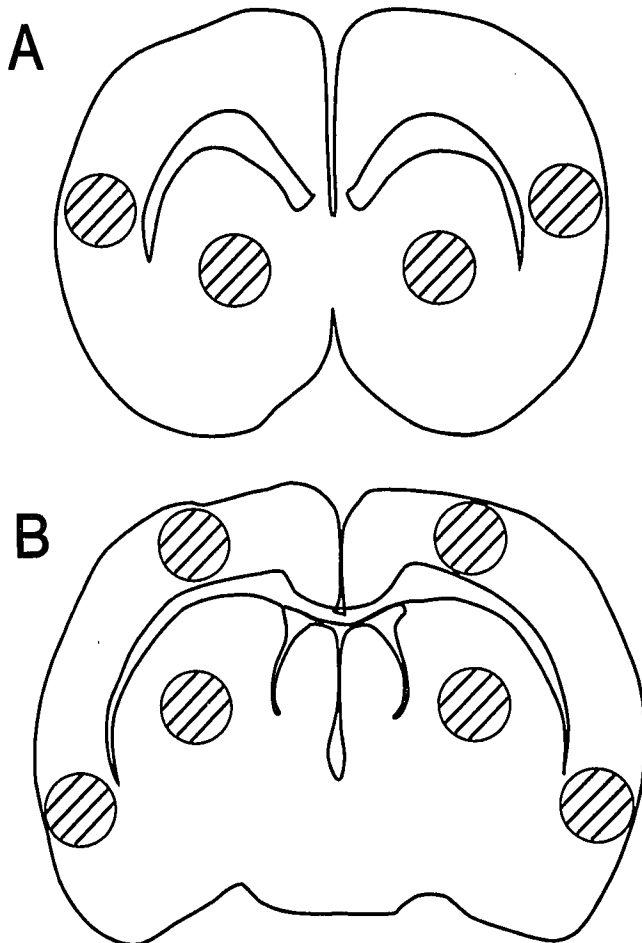


Fig. 3. Specific areas of tissue sampling for microgravimetry. (A) The posterior aspect of the 1-3-mm segment. (B) The anterior aspect of the 5-7-mm segment.

The TTC and microgravimetry data were not distributed normally and were assessed with the Kruskal-Wallis test with Dunn's test for multiple comparisons.^{29,30} $P < .05$ was considered significant.

Results

Part A

All data are presented as mean \pm SD. Except for hematocrit, there were no differences in the physiologic data (table 2). The phenylephrine dose required to maintain MABP at control levels (during MCAo) in the 30/albumin group was $4 \pm 2 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$. There were no abnormalities in TTC staining for the hemisphere contralateral to MCAo. In general, specific gravity was greater in the hemisphere contralateral to MCAo

versus the ischemic hemisphere. Infarct size was defined as the percent of cross-sectional area for the hemisphere ipsilateral to MCAo with deficient TTC staining (fig. 2).

In one of the two coronal sections, infarct size was less in the 30/albumin group versus the 44/B group. In both sections, infarct size was less in the 30/DCLHb group versus the 44/B and 30/albumin groups and in the 9/DCLHb group versus the other three groups ($P < .05$, table 3). As an example, in section 1 (3.0 mm from the frontal pole), infarct size was $44 \pm 4\%$ in the 44/B group, $37 \pm 3\%$ in the 30/albumin group, $31 \pm 3\%$ in the 30/DCLHb group, and $20 \pm 4\%$ in the 9/DCLHb group.

For all cortical areas in the ischemic hemisphere, specific gravity was greater (decreased brain water) in the 30/DCLHb and 9/DCLHb groups versus the 44/B group; and for two cortical areas, specific gravity was greater in the 30/albumin group versus the 44/B group ($P < .05$, table 4). There was no change in brain water content in the DCLHb groups versus the 30/albumin group.

Part B

There were expected between-group differences in hematocrit, total hemoglobin, and oxygen content (table 2).

Discussion

The results of this study support a hypothesis that, in general, hemodilution decreases focal cerebral ischemic injury and edema. In addition, if hemodilution is accomplished with an oxygen-binding fluid such as DCLHb, there is a dose-dependent effect of hemodilution on ischemic injury. The results also suggest that the ability of DCLHb to bind oxygen may convey an additional therapeutic effect on ischemic brain injury when compared to non-oxygen-binding fluids.

Hemodilution is postulated to augment CBF via two mechanisms: by decreasing viscosity or by a direct myogenic/neurogenic vasodilatory response to a reduction in oxygen content and delivery.³⁻⁵ The relative contribution of each mechanism may depend on whether hemodilution is employed in normal or ischemic brain. Although myogenic/neurogenic-induced vasodilatation may have a meaningful role in normal brain, where such physiologic responses are intact, it is likely that during ischemia these vascular responses are attenuated or abolished. In an ischemic vasculature,

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Table 2. Physiologic Data for Parts A and B

	44/B	30/Albumin	30/DCLHb	9/DCLHb
Part A				
pH	7.41 ± 0.02	7.40 ± 0.02	7.41 ± 0.01	7.41 ± 0.01
Pa _{O₂} (mmHg)	127 ± 20	128 ± 16	133 ± 14	127 ± 14
Pa _{CO₂} (mmHg)	38.5 ± 1.4	37.9 ± 1.2	38.0 ± 0.9	37.7 ± 1.3
MABP (mmHg)	129 ± 8	129 ± 7	130 ± 4	130 ± 4
Hematocrit (%)	44 ± 1	30 ± 1*	30 ± 1*	9 ± 1†
Glucose (mg/dl)	127 ± 14	121 ± 15	125 ± 12	130 ± 12
Part B				
Hematocrit (%)	44 ± 1	30 ± 1*	30 ± 1*	9 ± 1†
Total hemoglobin (g/dl)	14.6 ± 0.4	9.7 ± 0.4‡	13.0 ± 0.3*	9.6 ± 0.4‡
Oxygen content (ml/dl)	19.3 ± 0.4	12.9 ± 0.3‡	17.3 ± 0.3*	12.7 ± 0.4‡

Values are mean ± SD. pH, Pa_{CO₂}, Pa_{O₂}, hematocrit, and glucose were collected at 30-min increments throughout MCAo and reperfusion, and MABP was monitored continuously and recorded in 15-min increments. In Part B, hematocrit, total hemoglobin, and oxygen content were measured after stabilization at the target hematocrit.

44/B group received 8.0 ml of donor blood and hematocrit maintained at 44%; 30/albumin group received 10% albumin to maintain hematocrit at 30%; 30/DCLHb group received 10% DCLHb to maintain hematocrit at 30%; and 9/DCLHb group received 10% DCLHb to maintain hematocrit at 9%.

* *P* < .05 versus the 44/B group.

† *P* < .05 versus the other three groups.

‡ *P* < .05 versus the 44/B and 30/DCLHb groups.

with low flow and shear rates, any decrease in viscosity will effect a greater increase in CBF than in normal brain.^{31,32} Moreover, during ischemia with maximum vasodilatation, it is difficult to conceive that a reduction in oxygen content will induce further vasodilatation. This premise is supported by recent data, during hypotension, which demonstrate an attenuation of vascular reactivity to reductions in oxygen content effected by hemodilution.³ Other data support viscosity as the predominant mechanism that increases ischemic CBF during hemodilution.^{33,34}

A potential problem when developing our methodology was maintaining equal MABP between groups. Hemoglobin substitutes are known to increase blood pressure, possibly by binding nitric oxide.²⁵ As hypertension is known to decrease ischemia,¹² it was anticipated that, if DCLHb was administered in its customary regimen (bolus), we would not be able to discern if a change in infarct size was due to hemodilution or hypertension. Thus, a method of administering DCLHb without hypertension was required. We observed that, if DCLHb was given initially as an exchange transfusion, there was neither an immediate change in MABP nor a change during subsequent bolus administration. The beneficial aspect of the hemodynamic effects of DCLHb was that MABP did not decrease as hemodilution was instituted. Accordingly, MABP was not expected to be different between the 44/B group and the 30/DCLHb

and 9/DCLHb groups. However, it was anticipated that, in the 30/albumin group, MABP would decrease with hemodilution.¹² Thus, a small dose of phenylephrine was required (during MCAo) to maintain MABP at control values. The bulk of the available data suggest that the ability of phenylephrine to ameliorate cerebral ischemia depends on a systemic pressor effect and is not a direct drug effect on the brain.¹² Phenylephrine was not required during reperfusion, and there were no differences in MABP between study groups during

Table 3. Area (% of the cross-sectional area for the hemisphere ipsilateral to MCAo) That Failed to Stain with TTC (brain infarction, fig. 2) in Coronal Brain Slices 3.0 and 5.0 mm Posterior to the Frontal Pole (fig. 1)

	44/B	30/Albumin	30/DCLHb	9/DCLHb
3.0-mm section	44 ± 4	37 ± 3*	31 ± 3†	20 ± 4‡
5.0-mm section	42 ± 4	38 ± 3	27 ± 4†	18 ± 3‡

Values are mean ± SD.

TTC = 2,3,5-triphenyltetrazolium chloride; 44/B group received 8.0 ml of donor blood and hematocrit maintained at 44%; 30/albumin group received 10% albumin to maintain hematocrit at 30%; 30/DCLHb group received 10% DCLHb to maintain hematocrit at 30%; and 9/DCLHb group received 10% DCLHb to maintain hematocrit at 9%.

* *P* < .05 versus the 44/B group.

† *P* < .05 versus the 44/B and 30/Albumin groups.

‡ *P* < .05 versus the other three groups.

Table 4. Microgravimetric Data in Each Hemisphere

	44/B	30/Albumin	30/DCLHb	9/DCLHb
Section 1				
Cortex				
Ischemic	1.035 ± 0.003	1.040 ± 0.002*	1.042 ± 0.002*	1.040 ± 0.002*
Contralateral	1.046 ± 0.001	1.046 ± 0.001	1.046 ± 0.001	1.046 ± 0.001
Basal ganglia				
Ischemic	1.045 ± 0.001	1.044 ± 0.002	1.044 ± 0.001	1.044 ± 0.002
Contralateral	1.047 ± 0.001	1.047 ± 0.001	1.047 ± 0.001	1.046 ± 0.001
Section 2				
Cortex (superior)				
Ischemic	1.035 ± 0.002	1.041 ± 0.003*	1.042 ± 0.001*	1.042 ± 0.002*
Contralateral	1.044 ± 0.002	1.045 ± 0.002	1.046 ± 0.001	1.045 ± 0.002
Cortex (inferior)				
Ischemic	1.035 ± 0.002	1.037 ± 0.003	1.040 ± 0.002*	1.039 ± 0.002*
Contralateral	1.044 ± 0.002	1.044 ± 0.001	1.044 ± 0.002	1.044 ± 0.002
Basal ganglia				
Ischemic	1.042 ± 0.002	1.039 ± 0.003	1.043 ± 0.001	1.043 ± 0.002
Contralateral	1.046 ± 0.002	1.046 ± 0.002	1.045 ± 0.002	1.045 ± 0.001

Values are mean ± SD. Section 1 was 1.0–3.0 mm from the frontal pole, and Section 2 was 5.0–7.0 mm from the frontal pole.

44/B group received 8.0 ml of donor blood and hematocrit maintained at 44%; 30/albumin group received 10% albumin to maintain hematocrit at 30%; 30/DCLHb group received 10% DCLHb to maintain hematocrit at 30%; and 9/DCLHb group received 10% DCLHb to maintain hematocrit at 9%.

* $P < .05$ versus the 44/B group.

any 15-min increment of MCAo or reperfusion. Accordingly, for simplicity, the physiologic data was consolidated over the entire MCAo and reperfusion periods.

A limitation in the interpretation of this study is TTC stain. During normal aerobic metabolism, TTC is converted by mitochondrial oxidative enzymes to a formazan product that results in a deep red stain of the brain. However, with prolonged ischemia, these enzymes are rendered dysfunctional, and by effecting a failure of TTC conversion to its red derivative, a pale area of brain is visually identifiable (fig. 2). Although the delineation of normal from abnormal brain is distinct, the interpretation of the pale area as infarcted is not absolute. Although most of the pale area is undoubtedly infarcted, a portion of this area may have a potential for recovery.²⁶ Notwithstanding, the goal of this study was to analyze histologically the immediate effects of treatment after temporary focal cerebral ischemia. Accordingly, TTC stain was chosen to avoid extending the study period beyond 5 h (a necessary delay with conventional microscopy), which would have provoked additional brain injury and obscured the immediate effect of treatment on infarct size.

A previous study, in a similar model of focal cerebral ischemia, demonstrated a dose-dependent effect of hemodilution with DCLHb on ischemic CBF (the maximum decrease in ischemia occurred at a hematocrit of

9%).¹⁶ The purpose of the present study was to evaluate whether this decrease in ischemia correlated to a histologic endpoint and to compare the effects of DCLHb to a more conventional hemodiluting fluid such as albumin (the rationale for hemodiluting to a hematocrit of 30% with albumin and DCLHb).² When oxygen content is compared between these two groups (table 2), there are significant differences. In contrast (although not measured in the present study), these two groups were likely to have similar viscosity values.^{22,23} A 9% hematocrit group was included to assess the dose-dependent effects of hemodilution with DCLHb and to have a group to which oxygen content was matched to the 30/albumin group but viscosity was likely to be different.

Although the use of hemodilution in laboratory studies has had success, clinical trials have been inconsistent.^{6–13} Several explanations may account for this inconsistency. The first is the likelihood of a therapeutic window after the onset of ischemia, after which therapeutic maneuvers that augment CBF are not effective in limiting infarction.¹⁰ If hemodilution is instituted after this window, ischemic injury may have matured to a point at which therapeutic efficacy is absent and only detrimental side effects are manifest.^{14,15} In the present study, hemodilution was instituted prior to ischemia, maximizing therapeutic efficacy but limiting

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model relevance to circumstances in which hemodilution can be employed prophylactically (situations that convey a predictable risk of ischemia, e.g., carotid endarterectomy, cerebral aneurysm surgery). The second issue concerns potential adjunctive effects of hypervolemia. When hypervolemia and hemodilution are employed concurrently, hypervolemia may counter decreases in perfusion pressure associated with hemodilution.^{7,9} In addition, hypovolemia has been implicated as occurring in a subpopulation of stroke patients and exacerbating ischemic injury.¹³ Finally, there is the issue of decreased oxygen content when hemodiluting with non-oxygen-binding fluids. Such fluids place inherent limits on oxygen transport and, therefore, the effectiveness and magnitude of therapy.² In theory, hemodilution with oxygen-binding fluids may convey a unique advantage in the treatment of temporary focal cerebral ischemia.

In summary, during temporary focal cerebral ischemia, the effect of hypervolemic-hemodilution with a hemoglobin solution on ischemic injury was compared to albumin. The results support a hypothesis that hemodilution with DCLHb decreases focal cerebral ischemic injury and that this effect is dose-dependent. In addition, the results suggest that DCLHb is a more proficient hemodiluting fluid than albumin. The therapeutic benefit of the present treatment modality must be addressed in terms of functional outcome in a higher species before a more definitive statement can be made.

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