

Diaspirin Cross-linked Hemoglobin Improves Neurological Outcome Following Reversible but Not Irreversible CNS Ischemia in Rabbits

Mark P. Bowes, PhD; Kenneth E. Burhop, PhD; Justin A. Zivin, MD, PhD

Background and Purpose Hemodilution using modified hemoglobin solutions may reduce ischemic central nervous system injury. Purified diaspirin cross-linked hemoglobin (DCLHb) is a cell-free hemoglobin that is intramolecularly cross-linked between the two α subunits, resulting in enhanced oxygen offloading to tissues and increased half-life. In the present experiments, we evaluated the ability of DCLHb to reduce neurological damage in two rabbit stroke models.

Methods In a reversible spinal cord ischemia model, ischemia of the caudal lumbar spinal cord was produced by temporary occlusion of the abdominal aorta. In an irreversible model of cerebral ischemia, plastic microspheres (50 μ m) were injected into the internal carotid artery and lodged in the cerebral microvasculature. DCLHb was administered 5 minutes after initiation of ischemia as either a 10-mL/kg infusion, 10-mL/kg exchange transfusion, or a 20-mL/kg infusion. Con-

trol animals received human serum albumin that was oncologically matched to the DCLHb.

Results In the spinal cord model, DCLHb significantly increased the duration of ischemia required to produce permanent paralysis from 27.33 ± 8.71 minutes (mean \pm SD) in controls to 42.59 ± 10.10 minutes in the 10-mL/kg exchange transfusion group and to 40.82 ± 18.16 minutes in the 20-mL/kg infusion condition ($P < .05$). DCLHb did not significantly reduce neurological damage in the microsphere embolization model.

Conclusions These data suggest that cross-linked hemoglobin reduces neurological damage after reversible central nervous system ischemia and that this is not attributable to hemodilution or hypervolemia only. (*Stroke*. 1994;25:2253-2257.)

Key Words • hemodilution • hemoglobin • rabbits • neuroprotection

Cell-free hemoglobin solutions are potentially useful in stroke therapy because of their oxygen-carrying capability and low viscosity. Although the potential for cell-free hemoglobin to serve as a blood substitute has been acknowledged for many years,¹⁻³ interest in blood substitutes has recently increased. This may be attributed to a number of factors, including shortages of blood for transfusion, the necessity of type-matching whole blood, the risk of blood-borne infections such as the hepatitis and HIV viruses, the reduced viscosity of cell-free hemoglobin preparations, advances in blood substitute technology, and a growing number of uses for these solutions.

Hemoglobin may be extracted from erythrocytes but, without modification, is not the ideal medium for delivery of oxygen to tissues. In the human red blood cell, hemoglobin is accompanied by a cofactor, 2,3-diphosphoglycerate, that decreases the affinity of hemoglobin for oxygen, thereby permitting offloading of oxygen.⁴ Extracellular hemoglobin lacks this cofactor and therefore has an unacceptably high affinity for oxygen. Hemoglobin, a tetrameric protein composed of two α -globin and two β -globin subunits, rapidly decomposes into two $\alpha\beta$ dimers and is excreted by the kidneys,

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resulting in a short biological half-life. Although concerns have been raised over potential toxicity of cell-free hemoglobin in animal models⁵⁻⁸ and possible renal side effects in early clinical trials,^{2,9} more recent reports indicate that toxicity of hemoglobin solutions probably reflects endotoxin or lipid contaminants rather than a direct toxic effect of hemoglobin.¹⁰⁻¹²

Cell-free hemoglobin may be chemically cross-linked with bis(3,5-dibromosalicyl) fumarate, preventing its dissociation and modifying its oxygen affinity.¹³⁻¹⁷ The resulting diaspirin cross-linked hemoglobin (DCLHb) exhibits reduced oxygen binding affinity and lower renal excretion, thereby increasing half-life and enhanced tissue oxygenation.¹⁸⁻²⁰ Cross-linked hemoglobin is an effective blood substitute, capable of sustaining isolated perfused kidney²¹ and heart²² or supporting cardiovascular and renal function in vivo^{23,24} as well as improving survival in animal models of hemorrhagic shock and resuscitation.²⁵⁻²⁷ Many properties of these modified hemoglobin molecules have recently been reviewed by Keipert²⁸ and by Kaufman and Estep.²⁹

Hemoglobin solutions are considered potentially useful in ischemia because of their oxygen-delivering properties and low viscosity. Hemodilution with DCLHb decreased the number of regions of low cerebral blood flow and increased oxygen delivery after brief cerebral ischemia³⁰ and reduced brain injury and edema after reversible ischemia and reperfusion³¹ in spontaneously hypertensive rats. DCLHb also reduced cerebral edema and improved cellular oxygenation in a feline stroke model.³² Although these data are suggestive, there are

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From the Department of Neurosciences 0624, School of Medicine, University of California San Diego, La Jolla, Calif (M.P.B., J.A.Z.); and Baxter Healthcare, Blood Substitutes Division, Round Lake, Ill (K.E.B.).

Correspondence to Mark P. Bowes, PhD, Department of Neurosciences 0624, School of Medicine, University of California San Diego, La Jolla, CA 92093-0624.

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to our knowledge no data demonstrating an improved functional neurological outcome from ischemic central nervous system (CNS) injury with DCLHb administration. In the present experiments we have evaluated the ability of DCLHb, administered as either an intravenous infusion or an exchange transfusion, to improve neurological outcome after CNS ischemia in two stroke models differing in degree and pattern of blood flow reduction^{33,34}: a rabbit spinal cord ischemia model (SCIM) and a multiple cerebral embolism model (MCEM).

Materials and Methods

Hemoglobin Solution

DCLHb (10.3 g/dL) was prepared by the Baxter Healthcare Corporation. Cell-free hemoglobin was obtained from outdated human blood, cross-linked,³⁵ and rendered virus free by heat treatment.^{36,37} The DCLHb used in these studies was electrolyte balanced and had the following properties: pH 7.36 at 37°C, oncotic pressure of approximately 42 to 44 mm Hg (hyperoncotic with plasma), oxygen affinity of 32 mm Hg at 37°C and pH 7.4, and a viscosity of <1.5 centistokes at 37°C. Controls received electrolyte-balanced and oncologically matched human serum albumin (HSA).

Animals

Male New Zealand White rabbits (2 to 3 kg) were individually housed and provided with food and water ad libitum before surgery.

Rabbit Spinal Cord Ischemia Model

Rabbits were anesthetized with halothane. The abdominal aorta was exposed at the level of the renal arteries through a paramedial incision. Small-diameter plastic tubing was placed around the aorta just distal to the left (more caudal) renal artery. The ends of the tubing were threaded through a small plastic button and then through a plastic tube of larger diameter, forming a snare ligature. The incision was closed around the tube so that the free ends of the tubing were accessible externally. Animals receiving exchange transfusion had a carotid artery catheter placed to allow blood withdrawal. The right carotid artery was exposed, and the catheter (filled with heparinized saline and sealed with an injection cap) was inserted, oriented toward the heart, and secured with ligatures. The incision was then closed with the distal end of the catheter accessible externally. The rabbits were allowed to recover for at least 2 hours; they appeared to behave normally before initiation of ischemia. Aortic occlusion was performed by pulling and clamping the small tube around the aorta. (Complete paraplegia is observed in all animals within 3 minutes of occlusion.) Occlusion durations encompassing all grades of neurological outcome, from complete recovery to permanent paraplegia, were selected. At the end of the ischemic period, the tubing was released to restore flow through the aorta. The tubing was removed, and the abdominal wall was closed with wound clips. Animals were returned to their home cages and maintained for 4 days. Rabbits that died within this period were excluded to ensure that no animals with aortic thrombosis were included in the data analysis. Neurological function was evaluated at 1 hour, 18 hours, and 4 days after ischemia-reperfusion by two observers blinded to animal treatment and duration of ischemia. Animals were classified by the presence or absence of paraplegia. Paraplegic animals showed no motor response to noxious stimuli in the hindlimbs and were totally incontinent. Rabbits that were classified as not paraplegic were either normal or had some motor function of the hindlimbs, even if only barely detectable. Bowel and bladder function were variable. If it was difficult to

ascertain whether an animal was totally paraplegic, the animal was classified as paraplegic.

Conditions examined in this experiment were control (HSA 20 mL/kg; n=14), DCLHb 10 mL/kg (n=13), DCLHb 10-mL/kg exchange transfusion (simultaneous equal volume blood/DCLHb exchange; n=14), and DCLHb 20 mL/kg (n=16). HSA or DCLHb was initiated 5 minutes after ischemia and was infused at a rate of 1 mL/kg per minute so that the infusion was complete in either 10 or 20 minutes.

Multiple Cerebral Embolism Model

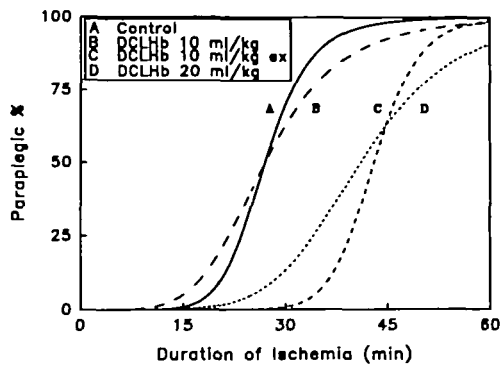
An incision was made lateral to the trachea to expose the right common carotid artery. The external carotid was ligated just distal to the carotid bifurcation. A catheter, filled with heparinized saline and sealed with an injection cap, was inserted antegrade into the common carotid artery and secured with ligatures. The incision was closed, and the distal end of the catheter was arranged to be accessible externally. The rabbits were allowed to recover from anesthesia for at least 2 hours; they appeared to behave normally before embolization. Plastic microspheres (50 μ m; New England Nuclear) were mixed with a tracer quantity of 15 μ m microspheres labeled with ⁵¹Cr. The mixture was carefully weighed to prepare the microsphere dose for each animal. The amount of radioactivity present in each microsphere dose was measured using a gamma counter. The microspheres were suspended in a solution of 0.05% polysorbate-80 in normal saline and transferred to 0.5-mL gas-tight syringes (Hamilton Co). The quantity of microspheres injected was varied from animal to animal to provide a range of microsphere doses encompassing all grades of neurological function. Animals were restrained briefly during embolization. The heparinized saline was withdrawn, and the microsphere suspension was rapidly injected into the carotid artery catheter. The injection system was flushed with 3 mL normal saline, carefully avoiding the formation of air bubbles within the catheter or injection cap. Animals were returned to their home cages for subsequent neurological evaluation.

Neurological function was evaluated 18 hours after embolization by two observers blinded to animal treatment and microsphere dose received. Each animal was classified as either functional (alert and able to right itself) or abnormal (dead or having unequivocal neurological deficit, such as greatly reduced level of spontaneous activity, inability to stand, or markedly uncoordinated movements). After evaluation, animals were killed with Beuthanasia-D (Schering-Plough), and their brains were removed. Radioactivity trapped in the brain was measured using a gamma counter. The total radioactivity recovered in the brain was compared with the specific activity of the injected microsphere dose to determine the weight of microspheres trapped in the brain.

Rabbits were randomly assigned to one of two conditions: control (HSA 20 mL/kg; n=11) or DCLHb (20 mL/kg; n=10). Treatment was initiated 5 minutes after embolization and infused intravenously at a rate of 1 mL/kg per minute so that the entire infusion was completed in 20 minutes.

Data Analysis

Neurological damage as a function of ischemic insult was analyzed using quantal dose-response analysis techniques described previously.^{38,39} A computer was used to fit logistic (S-shaped) curves to the fraction of abnormal animals as a function of ischemia duration (SCIM) or weight of microspheres trapped in the brain (MCEM). By using this technique, the ischemic duration or microsphere weight necessary to produce clinically apparent stroke in 50% of a group of subjects may be computed for each experimental condition (ET₅₀ in the SCIM, for effective time; ES₅₀ in the MCEM, for effective stroke). Pharmacological manipulations that improve neurological outcome will increase the ET₅₀ or ES₅₀, implying a shift of the dose-response curve to the right. Differences



Graph shows that diaspirin cross-linked hemoglobin (DCLHb) administration improved neurological outcome 18 hours after spinal cord ischemia-reperfusion. Each curve illustrates the increasing probability of permanent paraplegia associated with increasing ischemic duration. DCLHb significantly increased the duration of ischemia required to produce neurological injury in the 10-mL/kg exchange transfusion and 20-mL/kg infusion conditions but not in the 10-mL/kg infusion group. A similar pattern of results was observed at the 18-hour evaluation (see Table).

among the sets of control and treatment animals were compared using *t* tests with the Bonferroni correction for multiple comparisons, with $P < .05$ considered significant for all comparisons. This quantal bioassay technique allows the determination of dose-response curves with high efficiency using a limited number of subjects.

Results

Administration of DCLHb significantly reduced neurological damage resulting from spinal cord ischemia and reperfusion. At the 18-hour evaluation of SCIM subjects, the $ET_{50} \pm SD$ for the control group was 27.33 ± 8.71 minutes. The ET_{50} for the DCLHb 10-mL/kg infusion was 27.19 ± 13.98 , not significantly different from control. When 10 mL/kg DCLHb was administered as an equal-volume exchange transfusion, the ET_{50} increased significantly to 42.59 ± 10.10 minutes. Administration of 20 mL/kg DCLHb also significantly increased the ET_{50} to 40.82 ± 18.16 minutes. A similar pattern of results was observed 4 days after ischemia-reperfusion. These data are summarized in the Figure and in the Table. One animal in the control group died on the fourth day after occlusion; this animal was included in the 18-hour analysis but not in the 4-day evaluation.

In the MCEM, administration of 20 mL/kg DCLHb did not significantly improve neurological outcome. In control animals, the $ES_{50} \pm SE$ was 556 ± 138 μ g microspheres. In the DCLHb group, the ES_{50} was 595 ± 73 μ g microspheres.

Discussion

We evaluated the ability of DCLHb to attenuate functional neurological damage in two rabbit stroke models, one reversible and one irreversible. DCLHb significantly improved neurological outcome after reversible ischemia-reperfusion but not after irreversible ischemia induced by microsphere embolization. Neurological improvement was observed at both 18 hours and 4 days after ischemia-reperfusion, indicating that the improved neurological outcome is stable and persists after all of the DCLHb has been excreted. These data suggest that the effects of DCLHb do not reflect only

Effects of Diaspirin Cross-linked Hemoglobin on Neurological Status of Rabbits After Reversible Spinal Cord Ischemia

Evaluation	Group	n	$ET_{50} \pm SD$, min
18 Hours	Control	14	27.33 ± 8.71
	DCLHb 10 mL/kg infusion	13	27.19 ± 13.98
	DCLHb 10 mL/kg exchange	14	$42.59 \pm 10.10^*$
	DCLHb 20 mL/kg infusion	16	$40.82 \pm 18.16^*$
4 Days	Control	13	26.09 ± 10.88
	DCLHb 10 mL/kg infusion	13	25.52 ± 8.79
	DCLHb 10 mL/kg exchange	14	$40.06 \pm 7.96^*$
	DCLHb 20 mL/kg infusion	16	$43.82 \pm 19.52^*$

DCLHb indicates diaspirin cross-linked hemoglobin; ET_{50} , effective time to produce clinically apparent stroke in 50% of a group of subjects.

*Significantly different from control ($P < .05$).

hemodilution or hypervolemia because DCLHb-treated animals tolerated significantly more ischemia than controls receiving hemodilution with an equal or greater volume of HSA.

DCLHb significantly improved neurological outcome in the reversible SCIM but not in the irreversible MCEM. The SCIM may be a more sensitive test of neuroprotectant compounds because a difference in the survival of a small number of motor neurons may produce a detectable improvement in hindlimb function, whereas in the MCEM the survival of a large number of neurons may be required to detect neuroprotection. Alternatively, it is possible that in the MCEM, DCLHb sustains neurons and prevents the formation of infarction while the DCLHb is present in the system, but infarction develops after DCLHb has been cleared from circulation. This possibility is supported by the fact that histopathologic measurements taken shortly after ischemia-reperfusion demonstrate improved outcome with DCLHb.³⁰⁻³² A hemoglobin solution with a longer half-life may be required to produce lasting cerebral neuroprotection in this model. Hemoglobin solutions increase blood pressure (BP) in some species,^{26,40} possibly by upregulating adrenergic α -receptors,⁴¹ stimulating endothelin, and binding nitric oxide, thereby causing vasoconstriction.⁴² The effectiveness of DCLHb in the reversible but not in the irreversible ischemia model may reflect an enhancement of reperfusion caused by BP elevation in the reversible model, whereas the vasoconstriction or increase in BP may offset the supportive effects of DCLHb in the irreversible embolization model. We have observed a similar pattern of results with a neuroprotectant compound that also increases BP (M.P.B., unpublished data, 1994). An extensive body of literature on the spontaneously hypertensive rat (recently reviewed by Barone et al⁴³) demonstrates an increased susceptibility to CNS ischemic injury with elevated BP in these animals. Induced hypertension, however, improved cerebral blood flow and neurological outcome after reversible global CNS ischemia (induced by cardiac arrest) in dogs⁴⁴ and reduced brain injury after reversible ischemia-reperfusion in rats.^{45,46} Although the precise relationship

between BP and neurological injury is unclear, this issue is currently under examination in our laboratory.

A number of early reports raised questions regarding the potential toxicity of cell-free hemoglobin solutions. Toxic reactions have been reported in retina,⁶ liver,^{7,8} and kidney.⁸ More recent results indicate that these toxic reactions were attributable to inadequately purified or sterilized hemoglobin preparations.¹⁰ Przybelski et al¹¹ have used maze learning (water-alley maze and radial-arm maze) as an assay for DCLHb neurotoxicity in rats. Maze performance was not impaired, and heart, liver, and brain histology appeared normal after 20 mL/kg DCLHb. Thus, despite early concerns regarding the toxicity of hemoglobin solutions, it appears that DCLHb is without serious toxic effect at potentially useful doses.

In summary, the administration of DCLHb significantly improved neurological outcome after CNS ischemia-reperfusion in rabbits. These findings suggest potential therapeutic benefits in stroke, particularly in situations where blood flow may be restored (eg, as an adjunct to thrombolytic therapy).

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Editorial Comment

Diaspirin cross-linked hemoglobin (DCLHb) has had a long history of development as a potential blood substitute. This paper demonstrates the potential use of DCLHb in treating stroke by improving neurological outcomes after ischemia-reperfusion.

The development of a clinically useful blood substitute such as DCLHb has been hampered by several technical and environmental issues. First, red blood cell substitutes as commercial products are the first of their kind, and no definite guidelines or precedures are available for their clinical development. Every step of development breaks new ground.

Next, the AIDS epidemic has affected the world blood supply, and questions have been raised as to the availability of sufficient quantities of native adult hemoglobin that could be chemically converted to a cell-free blood substitute. Shortages in blood supply could be reflected as shortages of DCLHb and limit its availability. Technologies that use molecular biology to produce hemoglobin or those that use animal hemoglobins such as bovine hemoglobin would appear to have an advantage.

There has also been a continuing problem with the removal of endotoxins and lipid contaminants from blood substitute preparations. This problem should be solvable, but the cost factors involved in the purification step(s) may prove excessive.

Finally, defining a clinically measurable end point to demonstrate efficacy of a blood substitute appears to be an important criterion for Food and Drug Administration (FDA) approval according to a recent FDA workshop (Workshop on the Criteria for Efficacy of Red Cell Substitutes, January 11, 1994, held at the National Institutes of Health). Surrogate end points may not, by themselves, form the basis for approval, and the FDA appears to want more than proof of oxygen delivery as an efficacy end point. Blood substitutes such as DCLHb may present difficulties in this regard, especially in validating efficacy in the treatment of stroke.

Despite these difficult issues, the blood substitute community is in high gear, and some of the products should eventually appear for various therapeutic indications. The first applications for red cell substitutes such as DCLHb will probably be in the treatment of hemorrhagic shock and resuscitation or as a vehicle that can support cardiovascular and renal function, especially during surgery. If any of these applications prove beneficial, their use in the treatment of stroke will not be far behind.

Donald J. Abraham, PhD, Guest Editor
 Department of Medicinal Chemistry
 Medical College of Virginia
 Virginia Commonwealth University
 Richmond, Va

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M P Bowes, K E Burhop and J A Zivin

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