Hydroxyethyl Starch Reduces Leukocyte Adherence and Vascular Injury in the Newborn Pig Cerebral Circulation After Asphyxia

Stuart S. Kaplan, MD; T.S. Park, MD; Ernesto R. Gonzales, BSN; Jeffrey M. Gidday, PhD

Background and Purpose—Hydroxyethyl starch (HES) has beneficial effects on ischemic brain injury; however, its mechanism of action remains unclear. The present study was undertaken to test the hypothesis that HES can attenuate increases in leukocyte adherence and vascular permeability in the cerebral vasculature after global cerebral ischemia induced by asphyxia.

Methods—Pial venular leukocyte adherence and permeability to sodium fluorescein were quantified in anesthetized newborn piglets by in situ fluorescence videomicroscopy through closed cranial windows during basal conditions and during 2 hours of reperfusion after global ischemia induced by 9 minutes of asphyxia. Experimental animals received HES after the asphyxial insult (10% HES 257/0.47, 600 mg/kg IV bolus 5 minutes after asphyxia, followed by 600 mg/kg per hour IV drip during reperfusion; n=9).

Results—A progressive and significant (P<0.05) increase in adherent leukocytes was observed during the initial 2 hours of reperfusion after asphyxia compared with nonasphyxial controls. In this model, vascular injury, as determined by significant (P<0.05) increases in fluorescein permeability at 2 hours of reperfusion, is largely dependent on adherent leukocytes. HES significantly reduced (P<0.05) leukocyte adherence at 1 hour and 2 hours of reperfusion and reduced fluorescein permeability at 2 hours. HES did not change hematocrit or alter pial arteriolar diameter.

Conclusions—These findings indicate that a vascular anti-inflammatory action may underlie the beneficial effects of HES in global cerebral ischemia secondary to asphyxia. Since this compound is well tolerated by patients, future preclinical and clinical studies may reveal improvements in functional outcome with the early introduction of this or similar agents after perinatal asphyxia or global ischemia. (Stroke. 2000;31:2218-2223.)

Key Words: cerebral ischemia, global inflammation leukocytes reperfusion injury

Hydroxyethyl starch (HES) is a clinically well-tolerated complex polysaccharide that has recently been used in the therapeutic treatment of stroke and vasospasm after subarachnoid hemorrhage. It is available in multiple preparations, each with different pharmacological characteristics based on concentration, molecular weight, degree of substitution, and C2/C6 hydroxyethylation ratio. While HES has shown a protective effect with reductions in infarct size and/or improvement in outcome in experimental models of ischemic injury in both central nervous system (CNS) and peripheral tissues, results of its use in clinical stroke trials have been less encouraging. This may relate to the time window of administration of the compound relative to stroke onset, as well as the pharmacological characteristics of the particular HES preparation; nevertheless, clinical interest still remains, particularly in situations of elevated intracranial pressure.

The mechanistic basis of the beneficial effects of HES remains unclear. Postulated actions include improvements in cerebral blood flow, tissue oxygen delivery, and rheology. Recent in vitro and in vivo studies in peripheral tissues have demonstrated that HES can significantly reduce leukocyte-endothelial adherence. It is now recognized that cerebral ischemia/reperfusion leads to activation of leukocytes and cerebrovascular endothelium, and the resulting adherence of polymorphonuclear leukocytes to the microvascular wall contributes to reperfusion injury through the release of cytotoxic enzymes and the generation of oxygen free radicals. Whether or not HES affects leukocyte-endothelial interactions in the CNS microvasculature in the setting of cerebral ischemia, with potential implications for blood-brain barrier breakdown, is not known.
We have previously shown in newborn piglets that reperfusion after global cerebral ischemia or asphyxiasia is associated with significant and progressive increases in the number of leukocytes adherent to cerebral venules and a leukocyte-dependent increase in vascular permeability.21 We used the asphyxial model in the present study to test the hypothesis that HES would attenuate ischemia-induced increases in leukocyte adherence and vascular permeability and that the previously reported neuroprotective effects of this agent may be partially mediated by its anti-inflammatory actions.

Materials and Methods

Animal Preparation
Thirty-nine newborn piglets (age, 1 to 4 days) weighing between 1.5 and 3.5 kg were used in experimental protocols that were consistent with Public Health Service guidelines and approved by our institutional studies committee. The preparation for in vivo monitoring of leukocyte dynamics has been detailed previously.21 In brief, animals were premedicated with ketamine (20 mg/kg IM), tracheotomized, and mechanically ventilated with a mix of room air and oxygen with anesthesia maintained for the remainder of the experiment with isoflurane (1.0% to 1.5%). End-tidal CO2 and transcutaneous oxygen saturation were continuously monitored by a capnometer and forepaw sensor, respectively. Both femoral arteries were cannulated for continuous recording of arterial blood pressure and intermittent withdrawal of arterial blood samples for analyses of blood gases, glucose, and hematocrit (before ischemia and before the animals were killed). A femoral vein was cannulated for body fluid maintenance (6 mL/kg per hour of 5% dextrose in 0.45% normal saline) and paralysis (0.25 mg/kg per hour pancuronium). A thermo-regulated heating pad and overhead heating lamp were used to maintain core body temperature at 38°C to 39°C, the normal temperature for newborn piglets.

The animal was placed in a stereotaxic frame, and the head was held securely by ear bars placed against the mandibles. An 18-mm craniotomy was performed, the dura was removed, and a closed cranial window (12 mm) made of Plexiglas was mounted over the right parietal cortex just posterior to the coronal suture. The window was filled with artificial cerebrospinal fluid of a composition regulated heating pad and overhead heating lamp were used to maintain core body temperature at 38°C to 39°C, the normal temperature for newborn piglets. The window was filled with artificial cerebrospinal fluid of a composition and oxygen with anesthesia maintained for the remainder of the experiment with isoflurane (1.0% to 1.5%). End-tidal CO2 and transcutaneous oxygen saturation were continuously monitored by a capnometer and forepaw sensor, respectively. Both femoral arteries were cannulated for continuous recording of arterial blood pressure and intermittent withdrawal of arterial blood samples for analyses of blood gases, glucose, and hematocrit (before ischemia and before the animals were killed). A femoral vein was cannulated for body fluid maintenance (6 mL/kg per hour of 5% dextrose in 0.45% normal saline) and paralysis (0.25 mg/kg per hour pancuronium). A thermo-regulated heating pad and overhead heating lamp were used to maintain core body temperature at 38°C to 39°C, the normal temperature for newborn piglets.

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Epifluorescence Videomicroscopy

As in earlier studies,21 we used an epifluorescence microscope (model BHMJ, Olympus Corp) mounted on a position-flexible boom stand, with a 100-W mercury arc light source and a 3.3 photo eye piece. Two filter cubes were used. For imaging of rhodamine-labeled leukocytes, the excitation filter was 535/35 nm, the dichroic filter was 565 nm, and the emission filter was 610/75 nm. A standard fluorescein isothiocyanate cube, with excitation, dichroic, emission filters of 470/40, 505, and 510 nm, respectively, was used for imaging of vascular leakage of sodium fluorescein. The coupling of a 10× immersion lens (Olympus Corp) featuring a 0.4 numerical aperture and a 3.1-mm working distance with a Newvicon tube camera (Hamamatsu C2400) with contrast and brightness controls provided real-time, high-resolution images of individual fluorescently labeled leukocytes moving through the pial microcirculation on the surface of the brain. Final image magnification was ×290. Video recordings 30 seconds in duration were obtained at regular intervals (30 minutes) before and after asphyxia.

Leukocyte Imaging

Leukocytes were fluorescently labeled in situ by intravenous rhodamine 6G (R6G; Sigma Chemical Co), which stains 100% of circulating leukocytes as assessed by flow cytometry. In brief, 30 minutes before baseline measurements, a 2.5-mL/kg loading dose of R6G (60 µg/mL) was administered at 1.5 mL/min, and before each subsequent video image capture, a 1-mL maintenance dose of R6G (60 µg/mL) was infused at 1.15 mL/min via an automatic syringe pump to increase the fluorescence intensity of the leukocytes for ideal video recording contrast.

Protocols

Two baseline video recordings of leukocyte dynamics were obtained 30 minutes apart, followed within 5 minutes by the asphyxial stimulus, induced by turning off the ventilator for 9 minutes and clamping the respiratory tubing. A blood gas sample was obtained during the last minute of asphyxia, after which mechanical ventilation was resumed.

Animals were randomly divided into the following 3 groups: nonasphyxial controls (n=14), asphyxia alone (n=16), and asphyxia plus HES (10%; HES 257/0.47, Laevosan-Gesellschaft mbh; 600 mg/kg IV bolus 5 minutes after asphyxia followed by 600 mg/kg per hour drip during reperfusion; n=9). Leukocyte adherence and pial arteriolar diameters were measured at baseline and at 1 hour and 2 hours of reperfusion in each group. Vascular permeability was determined at 2 hours of reperfusion in animals from each group.

Quantification of Leukocyte-Endothelial Adherence

Leukocyte adherence to the endothelium of the pial venular wall was quantified in 2 preselected venular networks that included several secondary and tertiary (20 to 45 µm diameter) postcapillary branches and 1 or 2 larger venules (60 to 90 µm diameter) into which they drained. Adherence was quantified manually by counting the number of leukocytes that remained stationary anywhere within each venular network under observation for >10 consecutive seconds. The adherence values reported indicate the mean number of leukocytes per square millimeter of total endothelial vessel surface examined in the 2 networks, as determined by image analysis software (2-dimensional surface area times π[3.142]).

Quantification of Vascular Permeability

Leakage of sodium fluorescein (molecular weight=376; 0.55 nm radius) was determined at 2 hours of postasphyxial reperfusion by measuring perivenular increases in optical density 20 minutes after an intravenous dose of fluorescein. This was done in a cortical region separate from those used to measure leukocyte adherence to avoid potentially confounding effects of repeated imaging of the latter region. The methodology and quantification procedures have been described in detail previously.21 In brief, relative changes in perivenular optical density were measured from video records obtained 20 minutes after intravenous sodium fluorescein (1 mL/kg of a 0.04% sodium fluorescein in saline, administered over 1 minute). A minimum of 6 extravascular locations adjacent to pial venules were used; the 6 perivenular optical density values were typically similar and were averaged to obtain a representative value for fluorescein leakage in the pial venular network at that time. The diameter of these venules, as well as the length of the venules over which the leakage of fluorescein was assessed (400 to 900 µm), did not differ among the 3 animal groups.

Statistical Analyses

Differences in the physiological parameters within and between groups were assessed by repeated-measures ANOVA or nonparametric Kruskal-Wallis with Dunn’s or Dunnett’s multiple range test applied when appropriate. Differences in the hematological parameters and vascular permeability within and between groups were by paired
or unpaired Student’s t tests, respectively, with signed rank or Mann-Whitney rank sum tests as needed. P<0.05 was considered significant.

Results

Physiological and Hematological Parameters
There were no significant differences in the monitored physiological variables (blood pressure, blood glucose, arterial pH, \( P_{aco_2} \), and \( P_{ao_2} \)) among the 3 animal groups during baseline conditions or at 1 and 2 hours of reperfusion, and all values were within normal ranges for newborn piglets (data not shown). None of these parameters changed significantly over time in the control group. Asphyxial animals became severely hypoxic (\( P_{ao_2} = 14 \pm 2 \) mm Hg), hypotensive (mean arterial blood pressure \( = 22 \pm 5 \) mm Hg), acidicotic (pH \( = 7.10 \pm 0.04 \)), bradycardic (21 \pm 8 bpm), and hypercapnic (\( P_{aco_2} = 76 \pm 5 \) mm Hg) by the end of the 9-minute asphyxial insult; however, these parameters recovered to preasphyxial levels by 30 minutes of reperfusion. There were no significant differences in hematocrit between the level measured at baseline and that measured at 2 hours of reperfusion, within and between groups.

Pial Arteriolar Diameter
No significant change in pial arteriolar diameter occurred over time in the nonasphyxial control group. In animals rendered asphyxial, arteriolar diameters remained at preasphyxial baseline levels throughout the 2-hour reperfusion period. Posttreatment with HES also did not affect pial arteriolar diameter at any time.

Leukocyte-Endothelial Adherence
Under baseline conditions, no significant differences were noted among groups with respect to the number of leukocytes adherent to cerebral venules. In nonasphyxial control animals, a slight but significant increase in leukocyte adherence occurred over the 2-hour observation period relative to that measured during baseline conditions. However, as shown in Figures 1 and 2, asphyxia resulted in a much more robust and significantly greater increase in the number of leukocytes adherent to the venular endothelium during the initial 2 hours of reperfusion relative to time-matched controls. Animals treated postischemically with HES exhibited significantly less leukocyte-endothelial adherence at both 1 hour and 2 hours of reperfusion compared with asphyxial, time-matched controls.

Vascular Permeability
Figures 2 and 3 show that the pial venular permeability to sodium fluorescein was significantly elevated after 2 hours of reperfusion in the asphyxial animals relative to controls. In animals treated with HES, this asphyxia-induced vascular leak was significantly attenuated to levels equivalent to nonasphyxial controls. As shown qualitatively in Figure 2, in nonasphyxial animals, fluorescein lightly stained the venular endothelium and defined the vascular network against a darker background. In asphyxial animals, fluorescein appeared extravascularly, spreading away from the venules; this typical result caused the vascular network to appear dark against a lighter background. The fluorescence pattern in HES-treated asphyxial animals looked like that seen in nonasphyxial controls. Fluorescein leakage was not observed in the perivascular space adjacent to arterioles.

Figure 1. Reduction in the number of leukocytes adherent to cerebral venules during 2 hours of reperfusion after asphyxia in animals treated with HES (n=9) relative to that observed in untreated asphyxial animals (n=14) and nonasphyxial sham controls (n=14). \(*P<0.05\) vs respective value under baseline conditions; \(1P<0.05\) vs control group at same time; \#P<0.05 vs asphyxia group at same time.

Figure 2. Representative video prints to illustrate leukocyte adherence (left column) and venular sodium fluorescein leakage (right column) from animals in the nonasphyxial control group (top), the asphyxia group (center), and the asphyxia group treated with HES (bottom). Leukocyte adherence shown was measured at 2 hours of reperfusion. Fluorescein permeability, measured in a different cortical location, was quantified 20 minutes after an intravenous injection of sodium fluorescein at 2 hours of reperfusion (see Materials and Methods).
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with 6% HES 200/0.62 to a hematocrit of 30% resulted in a 

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reduce leukocyte-endothelial cell interactions promoted by 

Figure 3. Vascular fluorescein permeability, measured at 2 

hours of reperfusion and expressed as optical density units nor-

malyzed to background, in the 3 animal groups. The 81% 

increase in permeability that occurred in asphyxial animals 

(n=11) relative to time-matched controls (n=14) was blocked 

completely in asphyxial animals treated with HES (n=6). 

*P<0.05 vs control group at same time; †P<0.05 vs asphyxia 

group at same time.

Discussion

Accumulating evidence in peripheral vascular beds supports an inhibitory effect of a variety of complex polysaccharides on leukocyte-endothelial interactions. Ongoing studies in our laboratory21,23 indicate that, in this model of asphyxia-induced global ischemia, microvascular leakage of low-molecular-weight fluorescein is largely dependent on the magnitude of leukocyte adherence to venular endothelium. Consistent with these observations is our present finding that postischemic treatment with HES concomitantly attenuates increases in leukocyte adherence and vascular permeability during early reperfusion after global cerebral ischemia. This early improvement in microvascular integrity secondary to these anti-inflammatory effects of HES gives further impetus to continuing examinations of this agent in clinical stroke.14,15 particularly if study designs dictate that patients receive the agent early after stroke onset, which was not the case in previous trials.11–13

Studies in the peripheral vasculature indicate that HES can reduce leukocyte-endothelial cell interactions promoted by ischemia/reperfusion. For example, isovolemic hemodilution with 6% HES 200/0.62 to a hematocrit of 30% resulted in a 40% decrease in number of postischemic neutrophils adherent to skeletal muscle postcapillary venules at 30 minutes and 2 hours of reperfusion, but this effect was lost by 24 hours.16 In a porcine model of fecal peritonitis, resuscitation with pentafraction decreased the accumulation of neutrophils in the pulmonary capillary and hepatic sinusoids.17 The mechanistic basis of these antiadherent actions of HES is unclear and may include the reduction or modulation of the expression of adhesion molecules, including the selectins and/or integrins. Indeed, thrombin-stimulated human umbilical vein endothelial cells in vitro bound 229% more neutrophils than did similarly stimulated cells treated with pentafraction,18 which the authors attributed to a blunting of the increased adhesiveness of stimulated endothelial cells for neutrophils secondary to HES interfering with the binding of endothelial P-selectin with its sialyl Lewis$^*$ counterligand on the neutrophil. HES also prevented von Willebrand factor release in cultured human umbilical vein endothelial cells and inhibited early endothelial cell activation and rapid P-selectin expression, although no effect on endothelial E-selectin or neutrophil CD11b/CD18 expression was noted.19 Other studies suggest that HES may directly reduce the neutrophil chemotaxis through endothelial cell monolayers.24 These mechanistic pathways may underlie the reduction in leukocyte-endothelial adherence we noted in postischemic brain in the present study, but further experiments are needed to confirm in vivo these and other proinflammatory signaling pathways on which HES might exert influence.

HES molecules, particularly medium-molecular-weight fractions of pentastarch or pentafraction, reduce increases in microvascular permeability after ischemic insults in myocardium,9 gastrocnemius,10 and cremaster muscle.18,25 Studies in these noncerebral vascular beds suggest postulated mechanisms for HES-mediated reductions in edema, including the molecule acting as a seal between the capillary endothelial cell junction and the basement membrane.10,26 Such a mechanism is consistent with the HES-mediated attenuation of blood-brain barrier disruption caused by intracarotid hyperosmotic mannitol.27 HES may also reduce the transcytosis of serum proteins.4

The effect of HES on the postischemic CNS microvascu-

lature is less clear. Our study is the first to show a reduction in microvascular leakage in animals subjected to global cerebral ischemia secondary to asphyxia. Increases in microvascular permeability after focal brain ischemia2–4 or spinal cord ischemia1 were also reduced by medium-molecular-weight fractions of pentastarch or pentafraction. However, in models of focal embolic stroke28 and global ischemia,29 reductions in edema could not be demonstrated with low-molecular-weight hetastarch or pentastarch, respectively. These inconsistent outcomes could be due to differences in animal age and animal species used; different anesthetics, methods, and tracers used to measure vascular permeability; the time at which postischemic edema assessments were made; the dose administered as well as other intrinsic property differences between the various HES compounds30; and the extent to which HES and HES-like compounds affected hematocrit, blood viscosity, and autoregulatory capacity.30 Moreover, the type and severity of the cerebral ischemic insult and the associated degree of blood-brain barrier compromise, as well as the resultant physiological state of the brain, could also modulate the ability of HES to affect postischemic cerebrovascular integrity. The physiological and hemodynamic changes induced by asphyxia in our model are distinctly different from those induced by global ischemia by cardiac arrest, for example, and these changes may not only affect the integrated response of the tissue but the efficacy of therapeutic measures as well. Clearly, mechanistic studies are still required to elucidate how HES and related compounds can affect the specialized microvascula-

ture constituting the blood-brain barrier and reduce perme-

ability to solutes of all sizes.

Rheological factors and other indirect effects may contrib-

ute to the acute anti-inflammatory effects of HES that we
observed in the present study, such as a lowering of blood viscosity. However, hematocrit was unaffected at the concentration of HES used herein; thus, it appears unlikely that hemodilution played a significant role in reducing the post-ischemic inflammatory response in our study. We also cannot rule out the possibility that the HES-mediated reduction in postischemic leukocyte-endothelial adherence is secondary to an increase in cerebral blood flow, particularly an increase in venular blood velocity. However, the lack of change in the caliber of the resistance arterioles both during reperfusion after global ischemia and in response to HES administration argues against this mechanism, although absolute measures of cerebral blood flow are needed to conclusively address this possibility.

The important contribution of leukocytes to ischemic brain injury has become increasingly clear over the last few years. The present study supports the hypothesis that HES is capable of attenuating elevations in leukocyte adherence and leukocyte-dependent increases in vascular permeability in the newborn brain after an asphyxial insult. Although our previous study with a CD18 monoclonal antibody indicated some degree of dependence of fluorescein leakage on leukocyte adherence, it is likely that additional mechanisms, independent of an effect on adherent leukocytes, could contribute to the reduction in microvascular permeability resulting from HES administration. Moreover, our observations were made during the initial 2 hours of reperfusion; additional studies will be required using other models of ischemia/reperfusion to determine whether this anti-inflammatory effect is maintained over longer postischemic time periods and, ultimately, whether improvements in neuronal viability and functional outcome are realized. It is possible that the lack of significant efficacy in clinical stroke trials of HES is related primarily to the protracted time (48 hours) between ischemia and the initiation of therapy, although one recent study still found no treatment effect when HES was given within 6 hours of stroke onset. Other factors that might contribute to the demonstrated efficacy of HES in our and other preclinical studies relative to human stroke trials showing little or no benefit may also include differential alterations in volume status and differential effects of HES on coagulation and hemorheology, depending on the degree of substitution and the resultant in vivo molecular weight of the particular HES preparation. In any event, the present in vivo documentation of an anti-inflammatory action of HES reveals one mechanism whereby beneficial effects of this agent might be realized in the setting of global cerebral ischemia.

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**Editorial Comment**

Approximately 15 years ago, there was a great deal of interest in the use of hemodilution for acute stroke therapy. The fundamental concept at that time was that decreasing blood viscosity or increasing systemic blood volume might increase collateral flow to the margins of an ischemic cerebral zone and salvage some marginally viable tissue. Several clinical trials were conducted, but therapeutic efficacy was not proved. There were numerous possible reasons for these failures. At that time, it was common to randomize and treat patients many hours after the onset of symptoms. Another problem was that the animal model studies of hemodilution were conducted in young and healthy subjects, whereas the human stroke victims were suffering from a variety of cardiovascular problems in addition to their strokes, and congestive heart failure was a common side effect. Among the substances that were tested in these old trials were various starch preparations. Interest in these molecules then waned, and attention shifted to a variety of neuroprotective agents and thrombolytics.

Kaplan et al have a different idea. They show that hydroxyethyl starch (HES) reduces leukocyte adherence to pial venules and reduces fluorescein permeability into the brain parenchyma. Such antiinflammatory effects might reduce breakdown of the blood-brain barrier, microvascular occlusion, and transendothelial migration of leukocytes. Their study was focused on attempting to define the actions of HES on leukocytes after asphyxia and did not test whether the treatment actually reduced neurological damage. However, other studies have shown that HES can reduce infarct size and improve neurological function in experimental stroke models.

The classic stroke literature does not discuss the role of leukocytes in acute ischemic damage, because the obvious large parenchymal increases in leukocytes do not occur until several days after vascular occlusion. Thus, it was thought that these late white-cell accumulations simply were part of the process responsible for cleanup of necrotic debris. In recent years there has been much more interest in the early activation of leukocytes during ischemia. Leukocyte adherence to the vessel wall is triggered by ischemia, but this process is not usually the principal cause of necrosis. It, however, may be responsible for the no-reflow phenomenon that occurs when blood flow is restored in the large vessels but not in the microvasculature, and leukocyte activation may be responsible for some aspects of stroke-in-evolution. No-reflow is seen in many types of tissues that are exposed to transient ischemia and has been reported in stroke models for decades. It was thought to be essentially a laboratory curiosity until the advent of thrombolytic therapy for stroke. It is entirely possible that some of the poor outcomes or deteriorations after successful thrombolysis are due to the actions of leukocytes rather than rethrombosis or edema formation.

The article by Kaplan et al suggests some new thoughts about HES therapy. The initial trial of leukocyte inhibition for acute stroke therapy with an antibody to the intracellular adhesion molecule-1 (enlimomab) was unsuccessful. However, there are several possible reasons for this failure, including the fact that enlimomab is a murine antibody, and such complex molecules may have unpredictable actions in another species (people). HES is a much simpler molecule. Further investigations of HES at lower doses than were used in the previous clinical trials may be useful, particularly as an adjunct to thrombolytic therapy.

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