Enhancement of Retinal Recovery by Conjugated Deferoxamine After Ischemia–Reperfusion

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Purpose. Although toxic to the retina in its native form, the iron chelator deferoxamine (DFO) shows no apparent retinal toxicity when bound to hydroxyethyl-starch (HES). Conjugation of DFO does not alter its iron binding properties. Once bound, iron is no longer active in the production of toxic oxygen intermediates. This investigation seeks to determine whether HES-conjugated DFO (HES-DFO) protects against ischemia–reperfusion injury in the retina.

Methods. Retinal ischemia was induced in cats, pretreated with HES-DFO, using both the vascular ligation and the increased intraocular pressure models. Retinal recovery was monitored by electroretinography. Fundal fluorescein angiography was performed in treated and untreated animals after ischemia–reperfusion.

Results. Our results indicate that pretreatment with an intravenous bolus of HES-DFO, significantly enhances recovery of the postischemic b-wave and decreases fluorescein leakage after ischemia–reperfusion.

Conclusions. HES-DFO improves recovery of the neural retina after ischemia–reperfusion. It also maintains the integrity of the blood–retinal barrier. The protective effect of HES-DFO on the blood–retinal barrier is consistent with its intravascular confinement. That HES-DFO results in protection of the neural retina underscores the importance of the blood–retinal barrier as a mediator of ischemia–reperfusion injury. HES-DFO may have a role in the early management of ischemic retinal disease both as an iron chelator and as a blood–retinal barrier protector. Invest Ophthalmol Vis Sci. 1994;35:669–676.

Little is known about the relationship of iron chelation to retinal ischemia. This is due in part to clinical and experimental evidence of retinopathy induced by iron chelating agents such as deferoxamine (DFO).

The retina however, contains the highest concentration of long-chain polyunsaturated fatty acids in the body and is known to be highly sensitive to peroxidation. Ferric iron has been shown, in vivo and in vitro, to be a major catalyst to lipid hydroperoxide production in the retina. Several investigators have shown that exposure of retinal tissue to iron increases lipid peroxidation secondary to hydroxyl (OH•) and superoxide (O2•−) radicals. Consequently an iron chelator, lacking ocular toxicity, may prove useful in protecting the retina from lipid peroxidation associated with ischemia–reperfusion injury.

DFO is an iron binding compound derived from Streptomyces pilosus. It has a high affinity (Kd 10−31 M) and specificity for ferric ions. Once bound by DFO, iron is no longer reactive in free-radical producing reactions. DFO has demonstrated an ability to protect ischemic myocardium and may protect kidney and gut. Halliwell has recently concluded that the protective effect of DFO derives largely from its ability to inhibit iron-dependent free-radical reactions. Attempts to protect the brain from ischemic injury through iron chelation have failed, however.

In fact, this is not surprising: DFO has been shown to have cerebral toxicity. DFO has also been shown, in both clinical and experimental studies, to be associated with ocular toxicity. It occurs at high dosages (intravenous administration of greater than 200 mg/kg per day) and at lower doses, over prolonged treatment periods. The disturbances observed include blurring of vision; impaired peripheral, color and

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night vision; retinal pigmentary changes; cataracts after prolonged administration; diminished visual acuity, vision loss and altered electroretinograms (ERGs) and electrooculograms.21-26

High molecular weight iron-binding compounds, prepared by the covalent attachment of DFO to biocompatible polymers, have been described.27 A variety of biocompatible polymers such as dextran and hydroxethyl-starch (HES) have been covalently attached to the amino group of DFO.27 The high molecular weight chelators retain the iron binding properties of native DFO, but now have a prolonged plasma half-life and lack the hypotensive effect of the native compound.27

Good et al26 first described an albino rat model of DFO induced, toxicity of the retina. Using this model, Gehlbach et al28 have shown that conjugated DFO does not cause toxic ERG changes. Their data suggest that HES-conjugated DFO (HES-DFO) may be used to modulate iron mediated reactions in the eye, without ocular toxicity.28 Attenuation of ischemia–reperfusion injury by HES-DFO has not been previously described in the retina.

Clearly iron is important in initiating and catalyzing oxygen dependent tissue injury. HES-DFO, however, lacks vascular permeability and does not enter the tissue. This calls into question the etiology of a protective effect recorded from the extravascular, neural retina. One possible mechanism is that HES-DFO acts locally, scavenging iron from the intravascular space thereby limiting the free radical mediated damage occurring at the blood–retinal barrier (BRB). This in turn results in protection of the distant neural retina. One possible mechanism is that HES-DFO preserves BRB integrity, protecting it from damage typically incurred during ischemia–reperfusion. We propose that attenuation of ischemia–reperfusion injury in the neural retina derives from maintenance of the BRB rather than from a direct effect on neurons or glia.

METHODS

Animals

Thirty-four adult cats were selected to exclude disease and ocular abnormality. They were randomly assigned to one of two control groups (8 cats each) or one of two experimental groups (9 cats each) and were housed on a 12-hour light cycle. The animals in this study were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Anesthesia

The animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). Subcutaneous injection of heparin sodium (500 U/kg) and intramuscular injection of atropine sulfate (0.04 mg) was administered at the time of induction. Additional sodium pentobarbital (20 mg/kg) was administered during the course of the experiment as indicated by return of the ear-flick reflex. The corneas were topicaly anesthetized with Ophthetlic 0.5% (propamcaine-HCl, Alcaine, Alcon, PR) and the pupils dilated with cyclogel 1% (Cyclogyl, Alcon, PR). Body temperature was maintained at 38 ± 0.5°C. The animals were euthanized at the end of each experiment with an overdose of sodium pentobarbital.

The Models of Ischemia

All animals were subjected to 90 minutes of global ocular ischemia followed by 120 minutes of reperfusion. Two models of ischemia were used. In the first, eyes for vascular ligation underwent temporal orbitectomy combined with peri orbital stripping. At the section's end-point the globe remained in the orbit isolated on a pedicle consisting of the optic nerve, ophthalmic arteries and the venous outflow. A ligature placed around the pedicle initiated ocular ischemia. In the second model, the cornea of the contralateral eye was pierced by a 25-gauge needle positioned in the anterior chamber, lateral to the pupillary aperture. A manual syringe driver and pressure manometer were attached to the needle. Intraocular pressure was raised to 160 mm Hg by advancing the syringe drive mechanism and forcing sterile, nonpyrogenic, clinical grade 0.9% NaCl (Baxter, Deerfield, IL), into the anterior chamber. In all cases a flat ERG response was achieved. The fundi were observed to be pale, and vascular pulsations were absent.

Electroretinography

The eyes were dark adapted for 45 minutes. Flashes of 50 ms at 7.9 × 10⁴ cd/m² were used to produce saturated scotopic a- and b-wave responses using a standard optical bench.28 ERGs were recorded simultaneously from both eyes using silver-silver chloride–treated wick electrodes.28 Amplified signals were displayed on a Tektronix 5111 Storage Oscilloscope. Measurements were made from the stored signals as previously described.28

HES-Conjugated DFO

Thirty minutes before initiation of ischemia experimental cats received an intravenous injection of 10%
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HES-DFO in 0.9% clinical-grade NaCl solution (6 mg/kg) (Biomedical Frontiers, Inc. Laboratories, Minneapolis, MN). The result was a serum-conjugated DFO level (measured as DFO equivalents) at 1 hour of approximately 2 mM. (Serum concentrations determined spectrophotometrically by Biomedical Frontiers, Inc. Laboratories, Minneapolis, MN by the methods of Hallaway et al.27)

Each sample was collected at a designated time post injection, from a single individual. Each data point therefore represents a single serum DFO determination. The resulting vascular retention curve is comparable to that performed in mice by Hallaway et al.27 and in rats by Gehlbach et al.28 The limit of detection for the DFO assay is 50 μM. Blood samples were identified by random numbers blinding the laboratory to the order of collection.

Fluorescein Angiography

Fundal fluorescein angiography was performed on cats prebuffered with HES-DFO and on control cats, after 90 minutes of ischemia and 120 minutes of reperfusion. A Canon CF 60ZA (wide angle lens) fundus camera (Lake Success, NY) was used to photograph the fundus of animals injected with fluorescein (Fluorescite Injection, Alcon Labs Inc., Fort Worth, TX). Injections were made into the cat forearm vein 120 minutes after establishment of reflow. Photography was initiated 2 seconds after injection, and at 1-second intervals thereafter. (See Fig. 3 for photographs showing arterial and venous filling.) Late phase photographs were taken up to 20 minutes after injection of fluorescein. Work in this laboratory has confirmed that fluorescein angiography performed immediately upon reperfusion, results in substantially less leakage than it does after a period of reperfusion. A period of 120 minutes of reperfusion, after 90 minutes of ischemia, results in significant fluorescein leakage in both models of ischemia.

Data Analysis

In each animal the increased intraocular pressure model was applied randomly to either the right or left eye while the vascular ligation model was applied to the contralateral eye. Postischemic recovery data consisted of peak to peak b-wave amplitudes (a-wave trough to b-wave peak) recorded at scheduled time intervals after ischemia. The mean b-wave amplitude of all 34 cats used in this study was 457 μV. The interindividual error varied with a standard deviation of ±120 μV. The intraindividual error was less than ±10% for the typical animal as determined by replicate measures before initiation of ischemia.

Because of interindvidual variation, the absolute amplitude of each b-wave during recovery was normalized to the pres ischemic, b-wave control (amplitude of b-wave experimental − amplitude of mean control b-wave) × 100). For statistical treatment, the resulting recovery profiles from each experiment were subsequently averaged at each time point and plotted. This data was analyzed by the Macintosh statistical package StatView II, which performed unpaired t tests analysis at each time point. Each error bar in Figure 2 includes the data point ± a single standard deviation of error.

RESULTS

Serum HES-DFO levels measured as DFO equivalents show 2 mM peak serum levels at 1 hour. Levels remain above 1.5 mM throughout the duration of each experiment. Levels are 0.5 mM 45 hours after prebuffering (Fig. 1). Therefore a sustained level of HES-DFO is documented throughout the experimental period. As compared to native DFO, which has an intravascular half-life of 30 minutes, HES-DFO demonstrates prolonged intravascular retention.

Post ischemic recovery of the cat ERG b-wave after 90 minutes of global ocular ischemia is significantly improved by prebuffering with HES-DFO. Recovery of the ERG b-wave after reperfusion shows earlier and more complete recovery of the HES-DFO prebuffered animals in both the vascular ligation (Fig. 2A) and increased intraocular pressure (Fig. 2B) models of ischemia. Experimental animals demonstrate better recovery throughout the recovery period. Significance, (12% to 15% improvement), is achieved after 300 minutes in the vascular ligation model and
FIGURE 2. The recovery of the ERG b-wave plotted after 90 minutes of complete ocular ischemia. The ischemic challenge was administered using both the vascular ligation model (A) and the increased intraocular pressure model (B). Cats pretreated with HES-DFO (closed circles) are compared to cats without pretreatment (open circles). Data are summarized as the mean normalized b-wave at each time point, generating a typical recovery profile for each model. Error bars represent ± one standard deviation. The data points are fit by a second-order polynomial function with a coefficient of fit indicated by $R^2$.

after 330 minutes in the increased intraocular pressure model. Experiments were carried out until a $p<.05$ was achieved at the points of widest divergence between control and experimental recovery curves, (ie, 330 minutes and later). After significance had been achieved trend analysis indicated that the use of additional animals would result only in smaller standard deviations without substantially changing the relative or absolute position of the second-order polynomial curves, which described the data with coefficients of fit between 0.976 and 0.996.

Fluorescein angiography after 90 minutes of ischemia and 120 minutes of reperfusion demonstrates decreased BRB leakage in eyes pretreated with HES-DFO. Nonischemic control eyes show normal vascular filling (Fig. 3A). Fundi of eyes subjected to ischemia–reperfusion show dye leakage on reperfusion (Fig. 3B). Eyes prebuffered with HES-DFO and subjected to ischemia–reperfusion show reduced dye leakage on reperfusion (Fig. 3C).

DISCUSSION
Ischemia and reperfusion initiate an extremely complex cascade of biochemical responses in living tissue, which if sustained lead inevitably to tissue damage. The process is incompletely understood to date. Anderson, in his thesis, 29 has reviewed and organized many of the known reactions into an "ischemic cascade" of events. Specific cascade interventions have been proposed, for example, to encourage membrane stabilization, minimize edema formation, prevent vasospasm, block excitotoxin mediated cytotoxicity, maintain acid–base balance, manipulate substrate availability, oppose calcium and other ionic shifts, scavenge free radicals, and decrease oxygen consumption. 29 The result of intervention is an increased resistance to ischemia–reperfusion injury. A number of investigations have been directed at the various aspects of the ischemic cascade. For the purpose of this study the principal area of interest is the role of iron and iron derived oxygen free radicals in retinal ischemia–reperfusion.

That the iron chelator HES-DFO attenuates ischemia–reperfusion is consistent with the established finding that iron is important in initiating and catalyzing free radical reactions causing oxygen dependent tissue injury. The exact role of iron is not fully understood. It is clear however, that even submicromolar levels of nonheme iron support oxidative tissue damage. 30,31 Cyclical Fenton and Fenton-like reactions with $H_2O_2$, that produce highly reactive hydroxyl radicals ($\cdot OH$) are thought to drive the cytotoxic free radical reactions. 32

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^- \quad (1)$$

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2 \quad (2)$$
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Reaction 1 is the Fenton reaction. Reaction 2 describes Fenton cycling, where the ferric product is reduced back to the ferrous form by superoxide (O$_2$^-) or other single electron reductants.

Oxygen free radicals generated during ischemia–reperfusion readily attack the unsaturated bonds of free fatty acids in the lipid bilayer. This reaction can result in disruptions in the cell membrane leading to cell swelling, interstitial edema, cell death and tissue necrosis. Further evidence of free radical damage during ischemia–reperfusion is based on the finding that molecular oxygen reintroduced into ischemic tissue causes injury and that agents that scavenge or metabolize free radicals prevent injury.

Our investigation of the role of iron chelation in retinal ischemia–reperfusion injury, while based on the above, is further supported by the work of Li who has shown that DFO ameliorates retinal photic injury in albino rats, implicating iron in the production of hydroxyl radicals that mediate photic retinal injury. DFO may likewise be useful in protecting the retina from ischemia–reperfusion generated free radicals.

Our results indicate that HES-DFO attenuates ischemia–reperfusion injury in the cat retina. This finding is confirmed in the increased intraocular pressure and vascular ligation models. Experiments were conducted in both models as recent evidence suggests a pressure induced injury component in models of increased intraocular pressure.

Our data demonstrate a nearly equivalent net improvement of 12% to 15% in both models of ischemia, a finding consistent with gains achieved by other antioxidant regimens. In the vascular ligation model this improvement resulted in recovery of 90% of the control b-wave amplitude at 400 minutes. In the increased intraocular pressure model it represents 75% recovery. The difference in recovery between the two models supports the report of additional, nonischemic injury caused by models of increased intraocular pressure.

Fluorescein angiography performed on treated and untreated eyes results in diminished dye leakage across the BRB of HES-DFO treated eyes after ischemia–reperfusion.

The blood vessels of the retina do not allow passage of proteins and certain dyes into the interstitial fluid. This property is known as the BRB. The barrier is located at two levels: the retinal vascular endothelial cells and the retinal pigment epithelial cells, forming an inner and outer barrier. The anatomical determinants of both barriers are cell to cell tight junctions. The BRB can be disrupted by distention of the vessel wall, ischemia, damaged endothelial cells, and certain chemicals. HES-DFO is a large

FIGURE 3. Fundus fluorescein angiography of nonischemic control eyes with normal vascular filling (A). Fundi of eyes subjected to ischemia–reperfusion show dye leakage on reperfusion (B). Eyes prebuffered with HES-DFO and subjected to ischemia–reperfusion show reduced dye leakage on reperfusion (C).
molecule (average molecular weight = 100,000 Da) that does not cross the BRB. Its site of action is therefore confined to the intravascular compartment. This raises the intriguing question of how it protects the distant neural elements of the retina.

In studies of retinal ischemia in rats, periods of ischemia have been shown to increase the permeability of first the arterioles and later the veins. Experimental occlusion of the retinal veins has been shown to produce leakage from the venules. Westergaard et al have demonstrated increased permeability of Mongolian gerbil cerebral vessels induced by ischemia. No apparent damage to endothelial cells was detected. Reinecke et al demonstrated histologic evidence of endothelial cell damage in the retinal vasculature of cats, after 90 minutes of ischemia. No functional evaluation of vascular integrity was conducted, however.

In this study, we document preliminary evidence of functional compromise of the BRB in the cat after ischemia–reperfusion and propose that the BRB receives primary benefit from HES-DFO during reperfusion. We suggest that the actual protective effect derives from scavenging of iron in the intravascular space, at the BRB interface, thus limiting the catalysis of free radical production via Fenton Chemistry. Consequently, the now limited production of free radicals occurring at the time of reperfusion is handled by endogenous free radical scavenging systems. The result is limited peroxidation to the lipid bilayer of endothelial and retinal pigment epithelial cells and maintenance of their cell to cell tight junctions. Because the integrity of the BRB is preserved in treated animals, flooding of the extravascular compartment with the mediators of reperfusion injury is minimized. The improved recovery of the distant neural elements of the retina is therefore most likely the result of maintenance of extravascular, extracellular homeostasis at the time of reperfusion. The basis for this being preservation of BRB integrity. The angiography data presented here offers qualitative evidence in support of this finding.

Because ischemia without reperfusion is ongoing before reflow and because protection of the blood retinal barrier appears to limit the death of a profoundly ischemic neural retina, we suggest that conjugated forms of DFO may help to experimentally differentiate between retinal injury caused by substrate deprivation and that resulting from reperfusion. This idea emphasizes the importance of the role of the BRB in ischemia–reperfusion. Furthermore, the fact that elements of the neural retina are spared when reperfusion occurs in the presence of an intact blood–retinal barrier lends additional support to the idea that the constituent cells of an organ may be viable for extended periods of time when subjected to pure ischemia. Inability to salvage them may relate more to damage from reperfusion than it does to ischemic “starvation.”

Key Words
conjugated deferoxamine, ischemia–reperfusion, blood–retinal barrier, retina, cat, electroretinogram

References
17. Hernandez LA, Grisham MB, Granger DN. A role for iron in oxidant mediated ischemic injury to intestine.
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Am J Physiol. (Gastrointest Liver Physiol.) 1987;16:G49–G53.


