Polymer Conjugation Reduces Deferoxamine Induced Retinopathy In An Albino Rat Model

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Purpose. The iron chelating agent deferoxamine mesylate USP (Desferal, Ciba, Summit, NJ) is commonly used in the treatment of acute iron intoxication and chronic iron overload (associated with the transfusion-dependent anemias). When used for prolonged periods of time or in high doses deferoxamine is attended by a range of ocular toxicities. The visual symptoms associated with deferoxamine administration often limit effective iron chelation therapy and can result in permanent vision loss. Deferoxamine has recently been conjugated to certain high molecular weight biocompatible polymers without altering its iron-binding properties. Here the effect of conjugation of deferoxamine to hydroxyethyl starch on retinal toxicity is examined.

Methods. An albino rat model of electroretinographically determined, deferoxamine-induced retinal toxicity has been previously described. We use this model to evaluate and compare both native deferoxamine and hydroxyethyl starch conjugated deferoxamine.

Results. Our data show that retinal function, as assessed by the electroretinogram b-wave, is significantly depressed 1 day after a single dose of native deferoxamine, while the b-waves of rats receiving a single dose of hydroxyethyl starch-deferoxamine, are not significantly depressed at any time during the study. In addition, the administered dose of hydroxyethyl starch-deferoxamine resulted in plasma deferoxamine concentrations up to five times greater than those achieved with native deferoxamine.

Conclusion. These results suggest that hydroxyethyl starch conjugated deferoxamine is associated with less retinal toxicity than native deferoxamine and that it may be a safer alternative for iron chelation therapy. Invest Ophthalmol Vis Sci. 1993;34:2871-2877.

Deferoxamine (DFO), an iron-binding compound derived from Streptomyces pilosus has both 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 chelates iron from ferritin and hemosiderin, but poorly from transferrin and heme compounds. The chelate is water soluble and is cleared renally. Because of its iron-binding properties DFO is indicated in the treatment of acute iron intoxication and in the treatment of chronic iron overload due to transfusion-dependent anemias, such as β-thalassemia major. In the latter setting DFO slows the toxic accumulation of iron in critical organs and prolongs life.

The utility of DFO in iron overload states has been limited by its toxic properties. In the acute setting its inherent hypotensive effect limits the amount of drug that may be administered, (intravenous infusion not to exceed 15 mg/kg/hr or a total of 6.0 g in 24 hours). The chronic use of DFO is made difficult by its short intravascular half-life. Consequently DFO, which is not absorbed orally, must be administered by frequent intramuscular injection, continuous subcutaneous infusion, or slow intravenous infusion.

Administration of DFO is further complicated by ocular dysfunction. Toxicity is noted with administration at high doses, (intravenous administration of greater than 200 mg/kg/day), or prolonged administration. The ocular disturbances observed have included blurring of vision; impaired peripheral, color, and night vision; retinal pigmentary changes; cataracts after prolonged administration; diminished visual
acuity, vision loss, and altered electroretinograms (ERG) and electrooculograms.\textsuperscript{12-16} Patients receiving DFO therapy require serial monitoring of visual as well as auditory function.\textsuperscript{17}

High molecular weight iron-binding compounds, prepared by the covalent attachment of deferoxamine to biocompatible polymers, have been previously described.\textsuperscript{19} These high molecular weight chelators retain the iron-binding properties of native deferoxamine, but have a prolonged plasma half-life and lack the hypotensive effects of the native compound. A variety of biocompatible polymers such as dextran and hydroxethyl-starch (HES) have been covalently attached to the amino group of DFO.\textsuperscript{10} This conjugation has addressed some of the problems of DFO administration while leaving its iron-binding properties essentially unchanged.

Conjugation increases the plasma half-life of DFO greater than tenfold, hypotension is not associated with intravenous administration, and the LD\textsubscript{50} is greatly increased. In mice the LD\textsubscript{50} (based on DFO equivalents) is 4000 mg/kg for dextran-DFO compared to 250 mg/kg for free DFO. As a result mice given otherwise lethal doses of iron can be rescued by high doses of intravenously administered DFO conjugate.\textsuperscript{18} Importantly, in this murine model, nontoxic doses of the free chelator fail to prevent iron toxicity whereas higher doses are lethal.\textsuperscript{18} Finally the conjugates are effective in mediating in vivo iron mobilization and excretion.\textsuperscript{9}

Although the ocular toxic properties of HES-DFO have not been investigated to date, an albino rat model for DFO-induced retinopathy has been previously described by Good et al.\textsuperscript{19} The model was selected for use in this study because it has been shown to reproduce DFO induced retinotoxicity in a short-term model. Although the cause of DFO toxicity is incompletely understood, DFO does produce a rapid and reversible decrease in the b-wave of the ERG of the albino rat. This short-term toxicity does not occur in similarly treated rats of normal pigmentation.\textsuperscript{19}

In this study we first confirmed the toxic ocular changes in the ERG of DFO treated albino rats. We then examined the potential ocular toxicity of hydroxethyl-starch conjugated deferoxamine, (HES-DFO), in this model. Serum DFO levels are measured in each group of animals and are compared. Our data indicate that DFO and HES-DFO differ in their ability to induce toxic ERG changes and suggest that HES-DFO is a less retinotoxic form of iron chelator.

**MATERIALS AND METHODS**

Twenty-six male albino Wistar rats, aged 4–6 weeks, weighing 250–275 g, were maintained on a 12 hour:12 hour light/dark cycle. They received food and water ad libitum. Electroretinography was performed on the right eye of rats anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Corneas were anesthetized topically (proparacaine HCl; Ophthotic 0.5%, Allergan, Irvine, CA) and the pupils dilated (cyclopentolate HCl; Cyclogyl 1%, Alcon, Ft. Worth, TX). While anesthetized, rats were housed in a warmed cage, unanesthetized rats were housed at room temperature. Fifteen-minute recording sessions were performed at 23°C. Control and experimental recordings were performed under identical conditions.

ERG were recorded on a grounded bench. The active electrode was a silver silver-chlorided wick electrode that was connected via a standard Grass J-16 Photometer was used to measure light levels and resistance. While anesthetized, rats were housed in a warm cage, unanesthetized rats were housed at room temperature. Fifteen-minute recording sessions were performed at 23°C. Control and experimental recordings were performed under identical conditions.

Flash stimuli were provided by a standard optical bench containing a 100 Watt Tungsten-Iodide light source (6321, Oriel Co., Stratford, CT), heat and ultraviolet filters (Melles Griot, Glencove, NY), a 5-log unit, calibrated 6” crossed iconel wedge set (Eastman Kodak, Rochester, NY), a Uniblitz shutter (Vincent and Associates, Rochester, NY) and focusing lenses suitable to direct the light beam onto a fiber optic cable (Oriel #77800).

The albino rats were dark adapted for 30 minutes beginning at the same time each morning. A Tektronix J-16 Photometer was used to measure light levels and calibrate the optical bench. Ambient light levels were 0.1–0.5 cd/m\textsuperscript{2} throughout the experiment. Flashes of 50 ms at 7.9 x 10\textsuperscript{4} cd/m\textsuperscript{2} were used to produce saturated scotopic a- and b-wave responses. The light beam was directed incident on a maximally dilated pupil, illuminating primarily the central retina. Although a brighter flash predictably altered the ERG wave form, it did not produce a significant increase in the peak to peak amplitude of the recorded signal. A rat ganzfeld dome receiving a flash of equal intensity also failed to significantly enhance the peak to peak amplitude. The ERG were displayed on a storage oscilloscope from which the data were recorded. ERG were recorded at 2-minute intervals during a period of 12 minutes producing a total of 7 ERG.

The mean b-wave amplitude, (a-wave trough to b-wave peak), was the index used to assess retinal toxicity. The average b-wave amplitude was calculated as
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the mathematical mean of the b-wave peaks. On day zero, before treatment, the mean ERG and average b-wave amplitude were determined for each rat. These data served as the set of control values for that rat throughout the remainder of the experiment. The variability in the ERG response is represented by the standard deviation in the averaged signal. This calculation was performed by digitizing each millisecond of data, as it was recorded, using Labview data acquisition software (LabView 2 [National Instruments, Austin, TX]). The digitized signals were then transferred to an Excel spreadsheet (Microsoft® Excel [Microsoft Corp., Redmond, WA]), where routine averaging and error analysis formulas were applied. Graphics were generated using Cricket Graph (Computer Associates, San Diego, CA) software. All software was supported by a Macintosh II FX computer (Apple Computer, Cupertino, CA).

Rats were assigned to one of four treatment groups: saline control; vehicle control (HES); DFO; or HES-DFO. The three rats assigned to the saline control group were given a 1 ml subcutaneous bolus injection of 0.9% NaCl solution and served as absolute controls. The ERG of these rats were compared to those of 7 rats given a 1 ml, subcutaneous bolus injection of DFO, 150 mg in 1 ml of 0.9% NaCl. In a separate set of experiments done the next week, eight rats were injected with an intravenous bolus of 2 ml HES-DFO, (9.8 mg/ml DFO equivalents). Intravenous injection was performed through a 1.5-cm abdominal incision into the distal inferior vena cava with a 27-gauge needle. The ERG of these rats were compared to those of six rats injected similarly with 2 ml of HES, which served as vehicle controls.

Pharmaceutical grade Hydroxyethyl Starch (Dupont [Wilmington, DE] Critical Care) and Desferal were used in these experiments. HES-DFO was prepared by Biomedical Frontiers, Inc. (Minneapolis, MN). Serum DFO levels were measured in rats injected with DFO and in rats injected with HES-DFO. Blood samples were collected from rat tail veins (0.5 ml) and refrigerated with 50 ul of heparin before spectrophotometric determination of plasma levels by the methods of Hallaway et al.10 One sample was collected at a designated time postinjection from each rat to avoid significant volume change in individual animals. Each data point therefore represents a single serum DFO determination at a designated time postinjection. The resulting vascular retention curves are comparable to those performed in mice by Hallaway et al.10 DFO was not detectable in the serum of rats injected with saline or HES solutions. The limit of detection for the DFO assay is 50 /uM. Blood samples were identified by random numbers blinding the laboratory to the order of collection.

Once each rat received its assigned treatment, (saline control, DFO, HES, or HES-DFO), the mean b-wave amplitudes were measured on days 1, 2, and 5. These posttreatment mean b-wave measurements were compared to the pretreatment (day zero) mean b-wave and expressed as normalized values. Normalization was carried out by dividing the mean pretreatment b-wave amplitude by each of the mean posttreatment b-wave amplitudes and expressing the resultants as a proportion of the pretreatment measurement.

Figure 1. (A) A raw data set consisting of seven electroretinographic recordings taken during one recording session from a control rat. (B) The resulting mean electroretinogram from this typical data set. The mean a-wave is calculated to be −135 μV, the mean b-wave (a-wave trough to b-wave peak) is calculated to be 257 μV. The standard deviation of the data at the a-wave peak is ± 30 μV whereas the standard deviation at the b-wave peak is ± 31 μV. The standard deviation for each millisecond of data is included as an indication of the variability in the averaged response.
**RESULTS**

Figure 1 is an example of both the raw (Fig. 1A), and the average (Fig. 1B) ERG responses, which demonstrate the sensitivity as well as the variability in our recording system. The standard deviation of the averaged data is presented for each millisecond of the response. The resulting “standard deviation envelope” provides a quantitative indication of the intraindividual error involved in our recordings.

There exists a small incremental and cumulative light adaptation in these serially collected recordings that are separated by only 2 minutes. The control and experimental data have been collected on identical schedules so they have been subjected to identical adaptation and the mean responses are comparable. This figure is also representative of the actual (μV) amplitudes of the a- and b-waves recorded in this albino rat model.

The results (Fig. 2) confirm the DFO-induced, ERG b-wave reduction in albino rats, in addition to the reversible nature of this finding. When compared to saline-injected controls 24 hours after injection, DFO-treated rats showed a greater than 40% reduction in the mean b-wave amplitude ($P = 0.007$). The mean b-wave amplitudes of DFO-treated rats returned to control levels 48 hours after injection and continued at control levels throughout the experimental period. The suppression of the ERG b-wave 1 day after DFO injection is suggestive of reversible retinal toxicity and is consistent with the findings first reported in albino rats by Good et al.19

In Figure 3, ERG b-waves of rats treated with intravenous injection of HES-DFO are compared to b-waves of rats treated with HES (vehicle control). At no point in the study period does a statistical difference between these two groups become apparent. No evidence of retinal toxicity is detected. Although not directly comparable, neither of these groups was statistically different from the saline control performed in Figure 2.

Experimental serum levels of DFO are documented in Figure 4. Subcutaneous injection of rats with 150 mg of DFO in 1 ml of normal saline (600 mg/kg) generates a serum DFO level of 265 μM at 1 hour. Levels at 3 hours are less than the limit of assay detection (< 50 μM). Intravenous injection of rats with 2 ml HES-DFO (19.6 mg DFO equivalents; 78 mg/kg) produces a 1-hour serum level of 1119 μM, levels at 20 hours are 172 μM. It is apparent that rats treated with HES-conjugated DFO have been exposed to peak blood levels of DFO that are nearly five times as high.
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The electrophysiologic findings of Good et al (rejection of 150 mg DFO. The horizontal line at 50 μM represents the assay’s reliable detection limit for DFO. Assay accurate to ± 5%.

as those causing toxicity in the DFO-treated rats. Total periods of detectable DFO exposure are nearly ten times as long in the HES-DFO treated rats (≈30 hours) as compared to DFO treated rats (≈3 hours).

DISCUSSION

The electrophysiologic findings of Good et al (reversible b-wave depression after DFO administration in the albino rat) are consistent with the morphometric observations of Leure-du-Pree and Connors. They note that in pigmented rats significantly higher doses of DFO are required to induce histologic pathology (electron dense inclusions in the retinal pigment epithelium and often in the subretinal space) comparable to that sustained in albino rats. An additional finding is that at high doses the integrity of the photoreceptor outer segments are maintained in normally pigmented rats and are disrupted in albino rats.

Retinal toxicity associated with DFO administration is manifest by a number of visual parameters and is not an exclusive ERG finding. In humans the retinal signs have been described in detail and are well summarized and interpreted by Porter and Huehns as follows.

"There is night blindness, annular field loss with a geographically similar defect of dark adaptation together with electrooculogram and ERG abnormalities characteristic of degenerative disorders of retinal photoreceptors involving the rods. This, together with retinal pigmentation, makes the condition indistinguishable from retinitis pigmentosa. The loss of central acuity and colour vision with central scotoma with electrooculograms, ERG and visually evoked response (VER) abnormalities is characteristic of degenerations affecting the cones." 16

The fundal pigmentation is noted to be variable. Changes in the pattern electroretinogram and fundus angiography have also been documented.15

The ERG as a test of retinal and retinal pigment epithelium (RPE) function is valuable but has limitations. The ERG is primarily a test of retinal function with the a-wave representing photoreceptor activity and the b-wave representing bipolar and Müller cell activity. The c-wave, although not part of the clinical ERG, is a measure of RPE function. It is, however, a slow signal that is not as easily recorded. Although in general terms the clinical ERG describes the functioning of the inner retina without giving any direct information about the RPE, the ERG may become secondarily abnormal in the presence of severe RPE abnormality as documented in DFO toxicity. The electrooculogram is sometimes considered a more direct test of RPE integrity but it is not an ideal test in intact, chronic animals.

The therapeutic effect of DFO relates directly to its serum concentration and because the iron-binding properties of DFO are not affected by conjugation, an elevated serum level represents an increased iron-binding capacity. We have shown that the serum half-life of DFO is ten times longer for HES-DFO than for DFO (Fig. 4). Therefore, the relative lack of toxicity cannot be attributed to either a lack of iron-binding capacity or a short exposure period after intravenous administration. These findings suggest that the therapeutic index of HES-DFO may well exceed that of native DFO.

The rapid intravenous infusion of native DFO is known to induce serious hemodynamic effects (hypotension, tachycardia, and shock) in the acute setting. In this set of experiments native DFO was administered by the subcutaneous route to avoid any hemodynamic complications that might secondarily affect the ERG, for example, decreased ocular perfusion. Furthermore, we monitored the ERG of rats immediately after administration of both native and conjugated DFO and noted no significant change. We therefore see no evidence for ocular hypoperfusion in the period during which hemodynamic compromise would be expected, that is, the period of peak drug level. Moreover, intra-arterial blood pressure monitoring in dogs shows that the serum concentration of free DFO required to induce hypotension exceed the serum levels achieved in these experiments. Finally, all rats used in these experiments recovered from anesthesia fully and as expected with no behavioral evidence of cerebral hypoperfusion. HES-DFO has been shown to be well tolerated by the intravenous route.
It is postulated that DFO affects the retina either through direct toxic effects or by secondary mechanisms resulting from the chelation of iron or other trace metals, for example, zinc or copper. In accordance with these mechanisms, DFO must ultimately either cross the blood–retinal barrier or create a significant intravascular sink for iron or other metals. HES-DFO, at the doses administered in this experiment, caused both an increase in peak serum level and intravascular retention of DFO. It would therefore appear that, at the doses administered, HES-DFO represents a relatively greater intravascular metal sink than does native DFO. Accordingly, we cannot simply attribute the toxicity of native DFO in this model entirely to intravascular metal chelation.

Although conjugation of DFO to high molecular weight biocompatible polymers decreases its ability to escape the vasculature, evidence for an extravascular mechanism of native DFO toxicity continues to accumulate: In patients with siderosis who are treated with DFO, reduction of the b-wave correlates best with increases in blood–retinal barrier permeability, as occurs with the onset of the diabetic state. In DFO-treated rabbits, anesthetized with urethane (which opens the blood–retinal barrier) toxic ERG changes occur more rapidly. Bypassing the blood–retinal barrier by intravitreal injection of DFO causes rapid destruction of the eye. Finally, in patients being evaluated for DFO-induced cerebral toxicity DFO has been measured in the cerebral spinal fluid, documenting blood–brain barrier flux. Although increased intravascular retention may well account for the relative lack of ocular toxicity associated with HES-DFO, the large increase in the LD50 in mice and the lack of hypertensive effects in other animal models suggest that conjugated forms of DFO may also be inherently less toxic.

The retinal tolerance of HES-DFO in this study was demonstrated in healthy animals with no known pathologic breaches in the blood–retinal barrier. It is not known whether the permeability of the blood–retinal barrier to DFO in the albino rat is different from that of normally pigmented rats. We do not suggest that the diminished ocular toxicity of HES-DFO applies in situations of blood–retinal barrier compromise. Further tests are warranted in such models.

The results of this investigation suggest that the use of conjugated forms of DFO may limit ocular toxicity in patients undergoing high dose or long-term iron chelation therapy. The relative lack of HES-DFO retinal toxicity may relate to its improved vascular retention and suggests that the ocular toxicities associated with native DFO are at least in part related to an ability to cross the blood–retinal barrier. In keeping with the findings of this study, HES-DFO may be a better treatment alternative, both for acute iron poisoning and for chronic siderosis. High doses of intravenous HES-DFO are better tolerated, as they lack significant hemodynamic effects and have a longer intravascular half-life. Improved tolerance of DFO in the conjugated form may extend its clinical uses to modulation of other important iron dependent processes, for example, production of tissue damaging, toxic hydroxyl radicals in ischemia and reperfusion injury.

Key Words
deferoxamine, retinopathy, toxicity, ERG, albino rats

References
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