The Long-Acting Parenteral Iron Chelator, Hydroxyethyl Starch-Deferoxamine, Fails to Protect against Alcohol-Induced Liver Injury in Rats

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Abbreviations: ALD, alcoholic liver disease; ALT, alanine aminotransferase, DFO, deferoxamine; HES, hydroxyethyl starch.

ABSTRACT

We studied the effect of the long-acting parenteral iron chelator, hydroxyethyl starch deferoxamine (HES-DFO) on liver nonheme iron, lipid peroxidation and pathologic changes in the liver in the intragastric feeding rat model for alcoholic liver disease. Male Wistar rats (225–250 g) were fed liquid diet and ethanol for 2 months. In control pair-fed animals, ethanol was isocalorically replaced by dextrose. Two additional groups of animals (dextrose and ethanol fed) received HES-DFO (25 mg deferoxamine equivalents/kg, three times a week). The blood ethanol level in the ethanol-fed animals was maintained between 150 and 350 mg/dl. For each animal, the levels of hepatic nonheme iron, lipid peroxidation and pathologic changes were evaluated. Ethanol administration caused fatty liver, necrosis and inflammation. Addition of HES-DFO to the ethanol diet increased the severity of pathologic changes, particularly necrosis and inflammation. The nonheme iron in alcohol-fed animals was significantly higher (18.3 ± 4.3 μg liver) than in pair-fed dextrose controls (12.5 ± 1.5 μg, P < .05). Addition of HES-DFO significantly increased nonheme iron levels in the dextrose-fed rats (17.1 ± 2.0 μg/g, P < .02) but not in ethanol-fed rats (20.0 ± 2.0). Ethanol increased levels of conjugated dienes; these levels were not altered by HES-DFO. The most significant observations in this study were: 1) the higher hepatic nonheme iron content in ethanol-fed rats compared with pair-fed dextrose controls; 2) the absence of changes in hepatic nonheme iron levels or lipid peroxidation in ethanol-fed groups treated with HES-DFO; and 3) the worsening of liver injury in ethanol-fed rats by HES-DFO.

One of the mechanisms invoked to explain alcoholic liver injury is an increase in the formation of oxygen free radicals and lipid peroxidation (Cederbaum, 1989; Nanji et al., 1994; Reinke et al., 1987). Although the mechanisms involved in lipid peroxidation are not completely understood, iron, and especially catalytic iron, has been implicated as an initiator of lipid peroxidation (Bacon and Britton, 1990; Minotti, 1992; Minotti and Aust, 1989). Although alcoholism and ALD per se are not associated etiologically with marked hepatic iron overload, there is growing evidence that only mild degrees of iron overload are sufficient to enhance alcoholic-induced liver injury (Bonkovsky et al., 1996). Recently, Tsukamoto et al. (1995), with the intragastric feeding rat model for ALD, showed that when iron was supplemented in a high fat ethanol diet to produce only a slight increase in liver iron concentrations, there was a synergistic increase in levels of hepatic malondialdehyde and 4-hydroxynonenal in the liver. The increase in lipid peroxide levels was accompanied by enhanced severity of liver injury.

We have previously shown that in ethanol-fed rats, therapy with an oral iron-chelator, 1,2-dimethyl-3-hydroxypropyridin-4-one, led to a reduction in hepatic-free iron, lipid peroxidation and fat accumulation (Sadrzadeh et al., 1994). To follow up on these observations, the present study was designed to investigate the efficacy of a more powerful iron chelator, DFO, in preventing alcoholic liver injury. DFO is an extremely potent iron chelator which has been shown to be useful in the management of patients with iron overload (Hershko and Weatherall, 1988). DFO, however, has a very short half-life which severely limits its utility. Hallaway et al. (1989) have developed a technique of covalently attaching DFO by its amino group to various polymers such as HES rendering a high molecular weight compound (HES-DFO) with iron-binding properties virtually identical with free DFO. The half-life of HES-DFO is about 22 h.

We tested the effectiveness of HES-DFO in preventing liver injury in the intragastric feeding rat model for ALD (French et al., 1986; Tsukamoto et al., 1990). This model allows for the evaluation of therapeutic interventions on ethanol-induced pathologic changes in the liver. When rats are fed polyunsaturated fatty acids with ethanol, the animals develop pathologic injury which includes fatty liver, necrosis
and inflammation (Nanji et al., 1989; Nanji and French, 1989).

Materials and Methods

Animal model. The experimental animals were male Wistar rats weighing between 225 and 250 g (Harlan-Sprague Dawley, Indianapolis, IN). All animals were fed for 2 months by continuous infusion of a liquid diet through permanently implanted gastric cannulas, as described previously (Tsukamoto et al., 1990). The amount of ethanol was initially 8 g/kg/day and increased up to 16 g/kg/day as tolerance developed. Blood alcohol levels were maintained between 150 mg/dl and 350 mg/dl. At the time when the highest ethanol levels were achieved, the average caloric distribution for each nutrient was 25% of total calories as fat, 21% as protein, 12% as carbohydrate and 42% as ethanol. In control animals, ethanol was isocalorically replaced by dextrose. For rats treated with the iron chelator (HES-DFO), the dose administered was 25 mg/kg (expressed as DFO equivalents) given intraperitoneally three times a week for a 2-month period. HES-DFO was kindly donated by Biomedical Frontiers Inc. (Minneapolis, MN). Development and characterization of this iron chelator has been described previously (Hallaway et al., 1989). The content of bound DFO in the compound used was 15% (w/w) of the conjugated form. Both dextrose and ethanol-fed rats were treated with HES-DFO. All rats tolerated the three-times-weekly injections without complication. At sacrifice, the animals were anesthetized, and blood was drawn from the aorta for enzyme measurements. The liver was perfused with ice-cold, iron-free saline, cut into small pieces and frozen immediately in liquid nitrogen. All animals received humane care in accordance with the guidelines issued by the National Institutes of Health.

Liver pathology. At the termination of each experiment, a small piece of liver was removed and fixed in formalin. The samples were stained with hematoxylin and eosin for light microscopy. The pathologist who carried out the histologic analysis had no prior knowledge of the different experimental groups. The liver pathologic findings were scored as follows (French et al., 1986): steatosis (the percentage of liver cells containing fat): 1+, less than 25% of cells containing fat; 2+, 26% to 50%; 3+, 51% to 75%; and 4+, more than 75%, inflammation and necrosis: one focus/lobule 1; two or more foci/lobule, 2+. The total liver pathology score was calculated by adding the scores from each of the parameters. At least five fields in three different sections of the liver were examined.

Biochemical analyses. Ethanol was measured in the blood by the alcohol dehydrogenase method (Sigma Chemical Co., St. Louis, MO). Plasma ALT was measured by an automated method in routine use in our clinical laboratories. Conjugated dienes were measured according to the method of Recknagel and Glende (1984). Butylated hydroxy toluene (90 μM) was added to the homogenate to prevent lipid peroxidation during the procedure.

Nonheme iron was determined in liver homogenate, with ferene S, as an indicator with the molar absorptivity of 35,500 M⁻¹ cm⁻¹ at 594 nm (Artiss et al., 1982). The liver was homogenized in NaCl solution (7 mM NaCl/100 mg tissue) and centrifuged at 1000 × g for 10 min. The clear supernatant (150 μl) was mixed with dH₂O (150 μl) and 150 μl of thiourea/ascorbate solution (4.4% and 2.68%, in dH₂O). Trichloroacetic acid (150 μl of 40% solution) was added to the mixture, vortexed and centrifuged for 30–60 s. The supernatant (500 μl) was then mixed with 125 μl of fresh ferene S solution (35 mg ferene S in 10 ml of 50% ammonium acetate solution). The mixture was incubated at room temperature for 5 to 10 min, and the absorbance was read at 594 nm. Control experiments were carried out to ensure that the measured nonheme iron was not from nonspecific iron released from ferruginous compounds during the procedures.

DFO measurement in liver. Quantitation of HES-DFO in liver samples was accomplished spectrophotometrically by measuring the concentration of the iron-saturated form of the drug in deproteinized liver homogenates. A 10% homogenate of liver was prepared by weighing out a piece of liver weighing several hundred milligrams and then homogenizing the tissue in the appropriate volume of 76 mM sodium chloride to yield a final tissue concentration of 10%. Conversion to the iron-saturated (ferrioxamine) form was accomplished by addition of 100 μl of 100 mM ferrous sulfate solution to 500 μl of liver homogenate and allowing the mixture to stand for 45 min. The solution was then deproteinized by adding 30 μl of 100% (w/v) trichloroacetic acid, vortexing thoroughly and then centrifuging the sample for 5 min at 10,000 × g. The pH of the solution was then adjusted by adding 250 μl of 2.5 M sodium acetate to 400 μl of clear supernatant. A blank sample was prepared by use of 500 μl of water in place of the liver homogenate. The absorbances of the solutions were determined at 429 nm and the concentration of DFO calculated with a molar absorptivity of 2700 M⁻¹ cm⁻¹.

Statistical analysis. Results are presented as mean ± S.D. Differences between groups were evaluated by analysis of variance, and multiple comparisons were conducted with the Student-Newman-Keuls method.

Results

The weight gain and blood ethanol levels (range, 150–350 mg/dl) were not significantly different among the various experimental groups.

Animals treated with ethanol had a significantly greater (P < .01) degree of pathologic injury (pathology score, 4.4 ± 0.8; n = 5) than dextrose-fed controls (0.4 ± 0.2; n = 5).

Administration of HES-DFO with ethanol resulted in more severe injury (pathology score, 6.6 ± 0.5; n = 5) (P < .02 compared with the ethanol-treated group). In particular, the degree of necrosis and inflammation was greater in the HES-DFO-treated rats (table 1, fig. 1, B and C). The increased severity of liver injury was confirmed by measurements of ALT in plasma (fig. 2). HES-DFO did not enhance liver injury in dextrose-fed rats (pathology score, 0.9 ± 0.4).

The concentrations of liver nonheme iron (μg/g wet weight) in the different treatment groups are shown in figure 3. Ethanol feeding resulted in a significant increase in liver nonheme iron (18.3 ± 4.3 μg/g wet weight) compared with the dextrose-fed group (12.5 ± 1.5 μg/g wet weight).

Treatment with HES-DFO significantly increased (P < .02) liver nonheme iron in dextrose-fed rats (17.1 ± 2.0 μg/g liver). Administration of HES-DFO to ethanol-fed animals did not result in any further increase in nonheme iron levels (20.0 ± 2.0 μg/g liver).

The extent of liver lipid peroxidation, expressed as conjuga-

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<td>Fatty Liver (0–4)</td>
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<tr>
<td>Corn oil-ethanol</td>
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<td>3</td>
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* None of the dextrose-fed groups exhibited significant pathologic changes.
gated dienes, in the various experimental groups is shown in figure 4. Ethanol feeding resulted in 2-fold increase in conjugated diene formation compared with dextrose-fed controls. Treatment with HES-DFO did not increase conjugated diene levels in either dextrose-fed or ethanol-fed rats. Measurement of DFO levels in liver confirmed that DFO did indeed accumulate in the liver. The levels of DFO were not significantly different in the dextrose-HES-DFO group (92 ± 6 27 mg/g) than in the respective ethanol group (112 ± 6 15 mg/g).

**Discussion**

Iron within the liver is found in several biochemical forms such as ferritin, hemosiderin, heme and the putative "intra-
cellular low molecular weight" chelate pool (Voogd et al., 1992). Although the exact identity of this low molecular weight iron is not known, it is apparently bound to weak chelators such as AMP and ATP. In this form, iron is able to catalyze free radical formation and lipid peroxidation. Despite convincing clinical and experimental evidence for liver injury as a consequence of excess iron, the specific pathophysiologic mechanisms leading to hepatocellular damage are poorly understood (Bonkovsky, 1991; Pietrangelo et al., 1995). One of the suggested mechanisms leading to iron-mediated cell injury is membrane lipid peroxidation. The reaction of nonheme iron with molecular oxygen and/or hydrogen peroxide is currently envisioned as the most likely source of reactive oxygen species (Minotti, 1992). Relevant to the role of iron in ALD is the observation by Hulcrantz et al. (1991) who showed that incubation of hepatocytes with ethanol led to the generation of a lipid chemoattractant, the production of which was blocked by DFO. The investigators suggested a therapeutic role for iron chelation in ALD.

We have recently shown that treating ethanol-fed rats with an oral iron chelator resulted in lower levels of nonheme iron and fat deposition in the liver (Sadrzadeh et al., 1994). The iron chelator used was 1,2-dimethyl-3-hydroxyprid-4-one (L1) which is a member of bidentate orally effective iron chelators. These compounds have been shown to be effective iron chelators both in vitro and in various animal models (Brittenden, 1992). To follow up on this observation, we used a potentially more potent iron chelator DFO. The efficacy of DFO is limited because of its short half-life in plasma (Hershko and Weathers, 1988). In addition, chronic use of DFO is usually associated with toxicity. To increase its half-life, DFO has been covalently bound to biocompatible high molecular weight polymers such as HES. HES-DFO retains the equivalent iron-chelating properties of free DFO and exhibits lower toxicity (Hallaway et al., 1989). To our surprise, HES-DFO was ineffective in lowering hepatic levels of nonheme iron or reducing lipid peroxidation. Additionally, the severity of pathologic changes in the liver of rats fed HES-DFO and ethanol chronically was increased. Although the absence of change in lipid peroxidation in HES-DFO-ethanol-fed rats was not anticipated, this may not be surprising because of the lack of decrease in hepatic iron.

One possible explanation for the absence of decreased free iron and lipid peroxidation could be that the high molecular weight and size of the chelator may prevent its diffusion from the extracellular space into the intracellular compartment which is where most of the iron-chelating activity takes place. Although we were able to show that DFO was present in liver in HES-DFO-treated animals, the exact intracellular site where DFO accumulates is unknown. Another possibility is that, under certain conditions, DFO may independently stimulate lipid peroxidation. One of these conditions, relevant to alcohol-induced liver injury, is the presence of increased amounts of lipid in the liver. The amount of DFO required to inhibit lipid peroxidation is much higher when the lipid concentrations are increased (Braughler et al., 1988). Finally, the HES-DFO complex with iron may be more reactive than iron itself. Support for this hypothesis is provided by studies which show that DFO can react with superoxide anion radicals, resulting in the formation of a relatively stable nitroxide free radical (Davies et al., 1987; Morehouse et al., 1987). Our results which show an absence of protection against liver injury by HES-DFO are in contrast to the observations of other investigators who have used HES-DFO as an iron chelator. For example, HES-DFO administered over a 2-week period has been used successfully to delay the development of diabetes in BB rats (Roza et al., 1994). In rats in which diabetes was delayed there were also significantly fewer inflammatory cells in the pancreatic islets. In a model of ischemia-reperfusion injury, HES-DFO limited lipid peroxidation and severity of liver injury (Jacobs et al., 1991). In the ischemia-reperfusion experiment, the effectiveness of HES-DFO was assessed over a 24-h period. DFO has also been shown to be protective against other types of toxic liver injury. DFO given to rats 1 h before a toxic dose of acetaminophen decreased the mortality rate and the degree of increase in liver enzymes (Sakaida et al., 1995). The mechanism(s) by which DFO protected against liver injury involved a reduction in lipid peroxidation probably secondary to iron chelation.

In conclusion, we have shown that HES-DFO fails to protect against alcohol-induced liver injury. This observation does not, however, negate the possible use of iron chelators in ALD. HES-DFO may have been relatively inactive because of the nonpolar environment in which iron-mediated free radical injury was occurring. Furthermore, the large size of the molecule may have prevented access of DFO to the intracellular site where free radicals were generated.

References


