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Deferoxamine Preventable Hepatocellular Damage, Following Hemorrhagic Shock

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Running head: Hemorrhagic Shock

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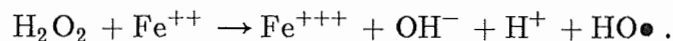
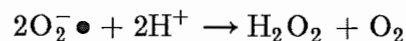
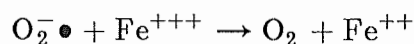
ABSTRACT

To examine the role of iron in mediating hepatocellular damage during reperfusion after hemorrhagic shock, we studied five groups of $n=4$ or 5 dogs, bled to reduce mean arterial pressure to 35 mmHg (± 5 mmHg) for three hours and subsequently treated 20 minutes prior to reinfusion of shed blood with either 0.9% saline, 5 ml/kg; deferoxamine, 50 μ g/kg in 0.9% saline; 6% pentastarch solution, 5 ml/kg; deferoxamine covalently bound to 6% pentastarch, 50 μ g/kg; or iron loaded deferoxamine in 0.9% saline. Saturation of iron binding capacity increased during hemorrhagic shock and remained high in all but the deferoxamine pentastarch treated group. Serum iron levels were significantly lower in animals treated with saline or iron loaded deferoxamine. Serum alanine aminotransferase (ALT), a specific marker for hepatocellular injury in the dog, was measured as an indicator of hepatic reperfusion injury. During shock, ALT did not change significantly from the normal in any group. After reperfusion, however, ALT rose sharply in animals reperfused without iron chelators, from a mean baseline value of less than 36 U/L to a post-reperfusion plateau of 5509 ± 2260 U/L at four hours, a pattern suggesting hepatic reperfusion injury. Iron loaded deferoxamine control dogs, also showed a substantial rise in ALT activity to 6287 ± 2134 U/L four hours after reperfusion. However, dogs treated with free deferoxamine showed significantly smaller elevations in ALT to only 655 ± 139 U/L ($P < 0.05$). Histologic evaluation of liver sections showed significantly smaller areas of necrosis in both of the deferoxamine treated groups compared to other groups. These findings suggest that deferoxamine preventable reperfusion injury of the liver, perhaps involving the iron catalyzed Haber-Weiss reaction, may play a role in the pathophysiology of hemorrhagic shock.

Keywords : iron chelators, lipid peroxidation, liver, oxidative stress, reperfusion injury

INTRODUCTION

Hemorrhagic shock frequently results in multi-system organ failure. Improved clinical management of renal and pulmonary complications following hemorrhagic shock has revealed the problem of shock-induced liver dysfunction.¹ One possible mechanism for hepatic dysfunction in shock involves damage by biological oxidants, including oxygen centered free radicals, hydrogen peroxide, and low molecular weight iron complexes. Such oxidative tissue damage following reperfusion of previously ischemic tissues -- so called reperfusion injury -- has been demonstrated in heart,² kidney,^{3,4} small intestine,^{5,6} skeletal muscle⁷ and liver.^{8,9} The most quoted chemical mechanism of oxidative stress in reperfusion is the iron catalyzed Haber-Weiss reaction* :



In this three reaction sequence redox cyclable, low molecular weight iron serves as a critical catalyst in the production of highly toxic hydroxyl radicals (HO•) from the less toxic bi-products of tissue oxidases, superoxide ($\text{O}_2^- \bullet$) and hydrogen peroxide H_2O_2 , now known to be liberated in increased amounts during the first few minutes of reperfusion after ischemia.¹⁰⁻¹² Iron within the hepatocyte may be present as transferrin-iron, ferritin, hemosiderin, or iron chelated to low molecular weight complexes.¹³ These physiologic chelators of iron are believed to make up the "transit" pool of iron, which permits the transfer of iron from the transport proteins to the stable storage forms. Because the liver is a major site of iron storage, it is also a likely site of iron mediated pathophysiology in hemorrhagic shock, perhaps because of shock induced release of toxic low molecular weight forms of iron from non-toxic high molecular weight complexes, such as

* Also known as the superoxide driven Fenton Reaction

ferritin.¹⁴ Many factors have been shown to influence the exchange of iron between the hepatocyte and plasma, such as an increase in extracellular levels of iron-transferrin,¹⁵ and elevations of extracellular "free" iron.^{15,16} Additionally, hypoxia has been shown to increase the hepatocellular release of iron *in vivo*, and experiments in hemorrhagic shock in intact animals have shown increases in serum iron.^{18,19} Recognizing such changes in iron levels, we surmised that the administration of an iron chelator during reperfusion therapy might be beneficial in diminishing the potential toxic effects of free iron, mediated by the iron catalyzed Haber-Weiss reaction.

The compound deferoxamine (Desferal, DFO) is a potent chelator of ferric iron²⁰ and is known to block the progression of the iron catalyzed Haber-Weiss reaction.^{21,22} Deferoxamine is currently used in human medicine to treat iron toxicity.^{23,24} It has also been shown to be beneficial to preventing reperfusion injury following CPR²⁵ and recent studies have shown that DFO treatment increases survival in dogs subjected to hemorrhagic shock.^{26,27} Deferoxamine is thought to enter cells by simple diffusion²⁸ and is then capable of chelating the "transit" pool of hepatocellular iron.^{28,29} Because of the short half life of DFO,²⁴ which may limit its effectiveness when given in a single dose, other investigators have examined the utility of deferoxamine covalently bound to pentastarch solution, both in hemorrhagic shock³⁰ and in ischemic/reperfused liver.³¹ The binding of deferoxamine to pentastarch solution prolongs its half life of deferoxamine without substantially altering its chelating ability.³²

The objective of the present study was to further examine the roles of iron and iron chelators in hepatocellular damage following hemorrhagic shock. Changes in iron status were studied by measuring total serum iron, unsaturated iron binding capacity, and total iron binding capacity. Both free deferoxamine and deferoxamine covalently bound to pentastarch were tested. Changes in alanine aminotransferase (ALT) a liver-specific enzyme, and changes in histopathologic appearance of liver tissue were used to assess hepatocellular damage. The results provided evidence of a surprising degree of deferox-

amine preventable and presumably iron mediated hepatocellular necrosis in the canine model of hemorrhagic shock.

METHODS

Animals

Following a protocol approved by the Purdue Animal Care and Use Committee, healthy dogs of either sex, ranging in weight from 10 to 17 kilograms, were pre-screened for abnormal blood chemistries and for heartworm disease and then anesthetized with pentobarbital sodium (30 mg/kg, i.v.). Additional pentobarbital anesthesia was administered, as needed, throughout the procedure. Both femoral arteries and one femoral vein were catheterized. One femoral artery catheter was used to bleed the animal, while the other was connected to a blood pressure transducer and physiograph stripchart recorder for continuous monitoring. Femoral vein catheter was used for return of shed blood and for drug administration. A lead II electrocardiogram was also connected to the physiograph to monitor heart rate.

The animals were allowed to stabilize for a period of thirty minutes, once all surgical procedures and connections had been established. Following the stabilization period three control blood samples for measurement of serum iron, unsaturated iron binding capacity (UIBC), total iron binding capacity (TIBC) and serum alanine aminotransferase (ALT) activity were taken at fifteen minute intervals (-30, -15 and 0 minutes). Heart rate and blood pressure were also recorded at these times.

Shock protocol

Hypovolemic shock was initiated at time 0 by blood withdrawal via a femoral artery for 10 minutes to achieve a mean arterial pressure (MAP) of 35 mmHg \pm 5 mmHg. The shed blood was stored at room temperature in a plastic container with acid-citrate-

dextrose solution (ACD)³³ added in a ratio of ACD to blood of 1:9. MAP was maintained at 35 mmHg \pm 5 mmHg, either by return of stored blood or by further blood withdrawal.

The dogs were randomly assigned to a treatment group at the start of each experiment. Treatment was started 20 minutes prior to reinfusion of shed blood at 3 hours after onset of shock with either 0.9% saline, 5 ml/kg (Group 1, n=4); deferoxamine (DFO, Cebia-Geigy Pharmaceutical, Summit, NJ), 50 mg/kg (Group 2, n=4); 6% modified pentastarch solution (MPS, Frontier Laboratories, Minneapolis, MN) (Group 3, n=5); modified pentastarch deferoxamine solution (MPD, Frontier Laboratories, Minneapolis, MN) 50 mg/kg (Group 4, n=4); and iron loaded deferoxamine (FeD), 50 mg/kg (Group 5, n=5). Iron loaded deferoxamine was made by combining deferoxamine with 10% molar excess of ferric-citrate (Sigma Chemical Company). Drugs were administered over a 10 minute period. Blood samples and recordings were then taken hourly for the next four hours. The animal was euthanized by intravenous injection of KCl; and liver samples were trimmed, fixed in neutral buffered formalin and processed routinely for light microscopic examination. Serum ALT levels were measured by Purdue University Veterinary Teaching Hospital clinical pathology laboratory (normal range: 20 to 125 U/L). Total serum iron, unsaturated iron binding capacity (UIBC) and total iron binding capacity (TIBC) were measured spectrophotometrically using Sigma's iron assay kit (#565-A).

Morphometry

Light microscopic morphometry was performed to measure the fractional area of hepatocellular necrosis in percent. Necrosis was identified by the presence of nuclear pyknosis, hypereosinophilia, and associated thrombosis of sinusoids. Slide labels were masked so that treatment or control group designations were unknown to the observer. The fractional area of necrosis was determined by a point counting method using a 100

point micrometer eyepiece for the light microscope. The numbers of points overlying necrotic areas in successive non-overlapping fields were recorded. The hematoxylin-eosin stained sections were examined at 4X magnification and scanned in 3.5 mm vertical increments at successive 2.5 mm horizontal increments from left to right. Such systematic sampling with a random start yields smaller errors than purely random sampling.³⁴

Statistics

Statistical analysis of the data included an initial test for homogeneity of variance by Bartlett's Chi-square method,³⁵ followed by an analysis of variance (ANOVA). If the variances of the compared data sets were not similar, a log transformation of the data was performed³⁶ and the ANOVA repeated on the transformed data. Specific comparisons between experimental and control groups were made with the Scheffe multiple comparison test. A P-value of 0.05 was considered significant.

RESULTS

The mean arterial blood pressure (MAP) for all groups during the control period ranged from 125 to 140 mmHg (Table I). The blood pressure in all groups returned toward normal following return of shed blood (99 - 119 mmHg) but then gradually decreased with time. The mean arterial pressures were not significantly different among the groups four hours after reperfusion.

Serum iron levels ranged between 138 and 502 $\mu\text{g}/\text{dl}$ for the thirty minutes prior to hemorrhagic shock (Table II). There was no statistical difference among the various groups during this period. As the period of hypovolemic shock progressed, there was a gradual increase in serum iron levels both in vehicle treated control groups and in the iron loaded deferoxamine group (Fig. 1). The free deferoxamine group showed a transient drop in serum iron immediately after infusion (3 hour reading) which returned to

pre-injection levels at 4 hours and then gradually decreased during the next three hours. Serum iron in the modified pentastarch deferoxamine group decreased immediately after infusion and remained low for the remainder of the experiment. Four hours after re-infusion there was no statistical difference between the free and conjugate deferoxamine treated groups. The lower initial serum iron level of the deferoxamine conjugate treated animals may indicate its confinement to, and concentration in the circulation, whereas free deferoxamine has a larger volume of distribution and lower serum concentration.

In an attempt to further examine iron levels, we measured UIBC and TIBC in the MPS, MPD and FeD groups. During the control period UIBC levels in all dogs ranged between 77 to 135 $\mu\text{g}/\text{dl}$. There was no statistical significance among the groups during this period (Table III). UIBC levels gradually and significantly decreased during shock in all groups (i.e. iron saturation increased). Following reperfusion UIBC levels in the pentastarch treated control group gradually increased with time (Fig. 2). UIBC in the iron loaded deferoxamine group increased at four hours then decreased over time. UIBC in the MPD treated group increased dramatically following reperfusion and remained elevated, indicating the expected addition of exogenous iron binding capacity. At three and four hours post reperfusion UIBC levels in the MPD group levels were significantly elevated compared the MPS and FeD treated groups, which were not significantly different from each other. Changes in the TIBC during the experiment are shown in Figure 3 and Table IV.

The ALT activities for all animals were within normal limits during the control period, ranging from 16 - 36 U/L (Table V). After three hours of hypovolemic shock there was a slight elevation in ALT activity, ranging from 55 to 1105 U/L, but there was no statistical significance among the groups. Serum ALT levels of all groups continued to rise dramatically over the next four hours, reaching levels 130 to 250 fold that of control, and indicating massive hepatocellular necrosis. The rise was greater for groups 1, 3 and 4 vs. group 2 treated with DFO (Fig. 4). As expected, iron loaded deferoxamine treat-

ment resulted in elevated total serum iron and iron saturation, with abolition of the protective effect of free deferoxamine. These results would indicate that it is the iron binding site of the deferoxamine compound that is providing protection and not some other chemical moiety such as the amine group.

Histologic examination of the liver sections showed that the majority of hepatocellular necrosis occurred was in the centrilobular regions with only minor changes in the periportal regions. The changes consisted of pyknosis, karyorrhexis, and moderate inflammatory cell infiltration. Figure 5 shows the fractional area of necrosis for animals in each treatment group. The saline group had the highest percent necrosis. The iron loaded deferoxamine group the second highest. The pentastarch bound deferoxamine and free deferoxamine groups had the least amount of necrosis. In addition, histologically observable necrosis in the free deferoxamine group was significantly less than than in the saline control, and the iron loaded deferoxamine group. Morphometrically, there was no statistical difference between the free and conjugate deferoxamine treated groups.

DISCUSSION

The present research provides provocative evidence that the multi-system organ failure in irreversible hemorrhagic shock³⁷ may in part result from reperfusion injury mediated by the iron-catalyzed Haber-Weiss reaction. This reaction has been proposed as the source of highly toxic and deleterious hydroxyl (HO•) or kinetically equivalent reactive intermediates^{38,39} capable of initiating lipid peroxidation as well as oxidative modifications of enzymes and nucleic acids that contribute substantially to cell death.⁴⁰ Recent studies with ischemic and reperfused liver have suggested a role for oxidative stress in hepatic necrosis. Allopurinol, an inhibitor of $O_2^- \bullet$ production by a xanthine oxidase can protect the liver from ischemia/reperfusion injury.^{41,42} Increased levels of hepatic malonyldialdehyde (MDA), a sensitive but non-specific indicator for oxidation of lipids, proteins, and nucleic acids, have been recently detected following hemorrhagic

shock.³⁰ Other studies have shown that iron is required for lipid peroxidation and subsequent breakdown of cell membranes.^{43,44}

The increase in serum iron that we observed (Table I) following hemorrhagic shock is in agreement with previous studies.^{18,27,30} However, the exact source of this iron is not known. Hypoxia has been shown to cause the release of iron from hepatocytes.¹⁷ The increase in iron saturation within the first two hours of shock (Fig. 2) also suggests that free iron is being released during the shock state, tending to saturate plasma iron carriers. The iron may be released from ferritin in the ferrous state by the action of superoxide and ascorbate.^{17,45} Such ferrous iron is capable of reacting directly with available H_2O_2 to produce $HO\bullet$ and ferric iron. Alternatively, ferrous iron may autoxidize to the ferric state and then be chelated by DFO, after which it is unable to catalyze hydroxyl radical formation.⁴⁶⁻⁴⁹

In our experiments the addition of deferoxamine significantly increased the UIBC of the plasma (Fig. 2), supplementing endogenous capacity for iron chelation. Therefore, the addition of the iron chelator is functioning not only to remove exogenous free iron from circulation but also to supplement the protective iron binding capacity of the serum.

The serum activity of the enzyme alanine aminotransferase was monitored because it is a liver-specific enzyme. ALT is found in the cytosol of the hepatocyte and is leaked into circulation with hepatocellular damage.⁵⁰ Because it is a cytosolic enzyme it may be released following damage to hepatocellular membranes. The early rise in ALT after one hour of shock may be due to hypoxia and subsequent leakage from the hepatocyte. The dramatic increase following reperfusion may be the result of cellular membrane damage from oxidative stress, i.e. reperfusion injury. In the present experiments the free deferoxamine significantly reduced the leakage of ALT following shock, however the deferoxamine conjugate did not provide the same degree of protection. This finding is in contrast to Drugas' results, in which the deferoxamine conjugate did reduce ALT leakage

from isolated ischemic and reperfused rat livers.³¹ In Drugas' experiments the deferoxamine conjugate was given at a dose of 100 mg/kg vs. 50 mg/kg in our experiments — perhaps indicating a dose or species effect. Similarly, a hemorrhagic shock model in pigs revealed that deferoxamine conjugate (270 mg/kg) decreased the release of aspartate aminotransferase (AST) 24 hours after shock.³⁰ Possibly a greater dose of the conjugated deferoxamine than was used in the present experiments would have been protective.^{30,31}

In toto, the present experiments strongly suggest that iron does play a role in the hepatocellular damage following hemorrhagic shock. Possibly "irreversible" hemorrhagic shock is caused in part by reperfusion injury mediated by iron and reactive oxygen species. We conclude the administration of deferoxamine, a strong iron chelator, does diminish serum iron levels, and that protection against biochemically and morphologically observed indicators of hepatic reperfusion injury damage can be obtained with deferoxamine treatment.

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TABLE I: Mean Arterial Pressure

Treatment	Pre-Shock	4 Hours
	(MAP) \pm S.E.	Post-Shock (MAP) \pm S.E.
SAL	140 \pm 14	109 \pm 15
DFO	125 \pm 7	80 \pm 9*
MPS	126 \pm 9	96 \pm 11*
MPD	130 \pm 12	87 \pm 12*
FeD	130 \pm 4	75 \pm 14*

* P < .05 pre vs post shock MAP

TABLE II: Serum Iron Levels

Treatment	Pre-Shock	3 Hours	4 Hours
	(μ g/dl) \pm S.E.	Post-Shock (μ g/dl) \pm S.E.	Post-Shock (μ g/dl) \pm S.E.
SAL	219 \pm 57	299 \pm 68	309 \pm 51
DFO	502 \pm 196	221 \pm 99	125 \pm 45#
MPS	138 \pm 23	151 \pm 42	184 \pm 34
MPD	147 \pm 20	16 \pm 7	10 \pm 9*
FeD	288 \pm 34	302 \pm 54	203 \pm 62

Note: DFO bound iron is not measured in the serum iron assay.

P < .05 vs. Saline treated

* P < .05 vs. all other groups except DFO group

TABLE III: Serum Unsaturated Iron Binding Capacity (UIBC)

Treatment	Pre-Shock	3 Hours	4 Hours
	($\mu\text{g}/\text{dl}$) \pm S.E.	Post-Shock ($\mu\text{g}/\text{dl}$) \pm S.E.	Post-Shock ($\mu\text{g}/\text{dl}$) \pm S.E.
MPS	135 \pm 25	72 \pm 27	86 \pm 25
MPD	81 \pm 11	485 \pm 9*	485 \pm 9*
FeD	77 \pm 28	29 \pm 16	25 \pm 15

Note: DFO iron binding capacity is measured in the UIBC assay.

* P < .05 vs. other groups shown

TABLE IV: Serum Total Iron Binding Capacity (TIBC)

Treatment	Pre-Shock	3 Hours	4 Hours
	($\mu\text{g}/\text{dl}$) \pm S.E.	Post-Shock ($\mu\text{g}/\text{dl}$) \pm S.E.	Post-Shock ($\mu\text{g}/\text{dl}$) \pm S.E.
MPS	275 \pm 8	241 \pm 13	270 \pm 16
MPD	228 \pm 12	498 \pm 7*	485 \pm 9*
FeD	365 \pm 38	331 \pm 45	310 \pm 79

Note: TIBC = serum iron + unsaturated iron binding capacity

* P < .05 vs. other groups shown

TABLE V: Serum Alanine Aminotransferase (ALT) Activity

Treatment	Pre-Shock (U/L) \pm S.E.	3 Hours Post-Shock (U/L) \pm S.E.	4 Hours Post-Shock (U/L) \pm S.E.
SAL	36 \pm 9	4223 \pm 1348	4585 \pm 1005
DFO	18 \pm 2	531 \pm 104*	655 \pm 139*
MPS	16 \pm 2	2493 \pm 555	3499 \pm 648
MPD	35 \pm 13	4370 \pm 1868	5509 \pm 2260
FeD	23 \pm 6	4717 \pm 1501	6287 \pm 2134

* P < .05 vs. other groups shown

FIGURE LEGENDS

Fig. 1. Mean serum iron levels ($\mu\text{g}/\text{dl}$) for saline (SAL), free deferoxamine (DFO), modified pentastarch (MPS), deferoxamine conjugated pentastarch (MPD), and iron loaded deferoxamine (FeD). All standard errors were less than 45% of the mean except saline group at 5 hours (52%).

Fig. 2. Mean serum unsaturated iron binding capacity (UIBC) levels ($\mu\text{g}/\text{dl}$) for modified pentastarch (MPS), deferoxamine conjugated pentastarch (MPD), and iron loaded deferoxamine (FeD). All standard errors were less than 32% of the mean except for 5 hour FeD (67%).

* $P < .05$ vs. other groups shown

** $P < .05$ vs. base line values

Fig. 3. Mean serum total iron binding capacity (TIBC) levels ($\mu\text{g}/\text{dl}$) for modified pentastarch (MPS), deferoxamine conjugated pentastarch (MPD), and iron loaded deferoxamine (FeD). All standard errors were less than 25% of the mean.

Fig. 4. Mean serum alanine aminotransferase (ALT) levels (U/L) for saline (SAL), free deferoxamine (DFO), modified pentastarch (MPS), deferoxamine conjugated pentastarch (MPD), and iron loaded deferoxamine (FeD). All standard errors were less than 40% of the mean except for 3 hour MPS, 67%.

* $P < .05$ vs. other groups shown

Fig. 4. Percent of necrotic area observed histologically for saline (SAL), free deferoxamine (DFO), modified pentastarch (MPS), deferoxamine conjugated pentastarch (MPD), and iron loaded deferoxamine (FeD).

