Combined Mannitol and Deferoxamine Therapy for Myohemoglobinuric Renal Injury and Oxidant Tubular Stress
Mechanistic and Therapeutic Implications

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Abstract
Mannitol (M) and deferoxamine (DFO) can each protect against myohemoglobinuric acute renal failure (MH-ARF). This study assessed M-DFO interactions during MH-ARF to help discern mechanisms of renal injury, and to define whether M + DFO exerts additive or synergistic antioxidant/cytoprotective effects. Rats subjected to the glycerol model of MH-ARF were treated with (a) M; (b) DFO; (c) M + DFO; or (d) no protective agents. Relative degrees of protection (24-h plasma urea/creatinine concentrations) were M + DFO > M > DFO > no therapy. To assess whether catalytic Fe is generated during MH-ARF, the bleomycin assay was applied to plasma/urine samples obtained 0–2 h post-glycerol injection. Although striking plasma and urinary increments were noted, excess renal hydroxyl radical (\(O^•H\)) production was not apparent (gauged by the salicylate trap method). M increased catalytic Fe excretion (four times), whereas DFO eliminated its urinary (but not plasma) activity. To determine direct M/DFO effects on proximal tubular cell oxidant injury, isolated rat proximal tubular segments (PTS) were incubated with toxic dosages of FeSO\(_4\) or H\(_2\)O\(_2\). Despite inducing cell injury (lactic dehydrogenase release), Fe caused no \(O^•H\) production. DFO conferred dose-dependent cytoprotection, correlating with increased, not decreased, \(O^•H\) generation. Although M scavenged this \(O^•H\) excess, it had no additive or independent, protective effect. H\(_2\)O\(_2\) cytotoxicity correlated with increased catalytic Fe (but not \(O^•H\)) generation. The fact that DFO (but not \(O^•H\) scavengers [M and dimethylthiourea]) blocked H\(_2\)O\(_2\) toxicity implied Fe-dependent, \(O^•H\)-independent cell killing. In conclusion, (a) striking catalytic Fe generation occurs during MH-ARF, but augmented intrarenal \(O^•H\) production may not develop; (b) DFO can block Fe toxicity despite a prooxidant effect; (c) H\(_2\)O\(_2\) PTS toxicity is Fe, but possibly not \(O^•H\), dependent; and (d) M does not mitigate oxidant PTS injury, either in the presence or absence of DFO, suggesting that its additive benefit with DFO in vivo occurs via a diuretic, not antioxidant effect. (J. Clin. Invest. 1992, 90:711–719.) Key words: iron • H\(_2\)O\(_2\) (hydrogen peroxide) • hydroxyl radical • myoglobin • proximal tubules

Introduction
In recent years, it has been demonstrated that the Fe chelator deferoxamine (DFO)\(^1\) can protect against myohemoglobinuric acute renal failure (MH-ARF)\(^1\)–3. Because Fe can promote hydroxyl radical (\(O^•H\)) production via the Fenton/Haber-Weiss reactions, it has been suggested that DFO’s protective influence could be caused by Fe chelation, leading to decreased \(O^•H\) production, and hence, less renal oxidant tissue stress. Thus, these results suggest that DFO could find clinical application for preventing MH-ARF.

Mannitol is another drug with proven efficacy against experimental (4–6), as well as clinical (7–9) heme protein nephrotoxicity. Like DFO, mannitol can also decrease \(O^•H\) levels, although it does so by \(O^•H\) scavenging, not by decreasing \(O^•H\) production. Furthermore, mannitol may protect against MH-ARF by its diuretic action, decreasing cast formation and possibly proximal tubular heme protein uptake (3, 4). Indeed, a recent study from this laboratory suggests that mannitol’s diuretic action could be primarily responsible for its in vivo protective influence (4).

Given that mannitol and DFO can each mitigate experimental MH-ARF, but that neither agent is totally protective (e.g., 1–4), an important clinical question is whether their benefits are additive. In this regard, the following issues seem relevant: (a) Because mannitol and DFO may confer protection by different mechanisms (diuresis and Fe chelation, respectively), their beneficial effects may be additive or synergistic. Alternatively, if mannitol and DFO each act by decreasing \(O^•H\) levels, then combined therapy may be superfluous. (b) Although DFO is generally believed to decrease Fe-mediated \(O^•H\) production, it can also paradoxically accentuate it, presumably by accelerating Fe\(^2+\) autoxidation (10, 11). Under this circumstance, provision of an \(O^•H\) scavenger such as mannitol might negate this adverse DFO effect, thereby enhancing its cytoprotective influence. (c) Because diuretics can theoretically decrease intrarenal DFO accumulation caused by enhanced urinary washout, mannitol might negate DFO’s protective influence. Alternatively, mannitol and DFO might work in concert to decrease the intrarenal Fe burden by exerting combined diuretic and chelation effects.

Given the above considerations, it is unclear whether mannitol and DFO have additive/synergistic or mutually exclusive protective effects against MH-ARF. Since this issue has not been previously tested, and because of its obvious clinical rele-

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\(^1\) Abbreviations used in this paper: ANOVA, analysis of variance; BUN, blood urea nitrogen; DFO, deferoxamine; DHBA, dihydroxybenzoic acids; DMTU, dimethylthiourea; LDH, lactic dehydrogenase; MH-ARF, myohemoglobinuric acute renal failure; \(O^•H\), hydroxyl radical; PTS, proximal tubular segments.
rance, the goals of the present study were to: (a) determine whether DFO addition to mannitol therapy can improve upon the latter's protective influence in an in vivo model of MH-ARF; (b) test the hypothesis that excess catalytic Fe (i.e., non-protein bound Fe capable of catalyzing free radical reactions) is, in fact, generated during MH-ARF; (c) assess whether this increased catalytic Fe activity causes excess intrarenal $^1$OH production; (d) determine whether mannitol and DFO decrease this catalytic Fe burden by enhancing its excretion and by chelation, respectively; (e) define whether mannitol and DFO confer direct and additive cytoprotection against proven proximal tubular oxidant insults (Fe and $H_2O_2$); and (f) ascertain whether such protection correlates with reductions in $^1$OH production (DFO) or with $^1$OH scavenging (mannitol). If so, these data, in concert, would support the hypothesis that mannitol and DFO exert additive protection in vivo because of overlapping antioxidant, cytoprotective effects.

Methods

In vivo experiments: effects of DFO on mannitol-mediated protection against MH-ARF. Male Sprague Dawley rats 200–330 g (Harlan Sprague Dawley, Indianapolis IN), maintained under standard vivarium conditions with free food and water access were used for all experiments. On the morning of experimentation, they were anesthetized with pentobarbital (30–40 mg/kg i.p.), two jugular venous catheters (PE-50) were inserted, and then each rat was injected intramuscularly with 50% glycerol, 10 mg/kg (two equally divided doses into each hind limb). After glycerol injection, the rats were divided into two experimental groups (a) in mannitol therapy/no DFO, 10 rats were infused with 1.25 ml of 25% mannitol per 100 g of body weight over 2 h, the first 1 ml being administered during 5 min at the start of the infusion; and (b) in mannitol plus DFO therapy, the rats received mannitol as noted above, with deferoxamine (Ciba-Geigy, Summit, NJ) being added to the mannitol infusion (total dose. 12 mg/100 g body wt). Urine was collected every 30 min 0–2 h post-glycerol injection by external bladder compression. The volume of urine passed was determined and then an equal amount of 0.45% NaCl was infused intravenously over 5 min through the second jugular venous catheter to avoid volume depletion caused by the mannitol-stimulated diuresis. The 2-h urine samples were saved for catalytic Fe assay, as described below. Then the rats recovered from anesthesia, free food, and water access being allowed. 24 h later, they were reanesthetized and killed by aortic puncture. The plasma was used for "blood" urea nitrogen (BUN) and plasma creatinine assay. The kidneys were resected and frontal sections were cut and fixed by immersion in 10% buffered formalin for subsequent histologic analysis, as described below.

As points of reference for the above experiments, the severity of ARF induced by glycerol injection alone and DFO's independent effect on it were assessed. To these ends, 16 rats were anesthetized and injected with glycerol as noted above. Half received a 2-h DFO infusion (total dose, 12 mg/100 g body wt, dissolved in 1 ml of 5% dextrose and water, the first 0.25 ml being administered over 5 min immediately after glycerol injection). The other rats received a sham DFO infusion. As detailed above, all urine excreted 0–2 h after glycerol injection was collected and saved for catalytic Fe assay. To correct for possible differences in urine output, and hence, extracellular fluid volume, the amount of urine passed each 30 min was determined and an equal amount of 0.45% NaCl was injected intraperitoneally (± 0.7 ml per injection). The rats were allowed to recover from anesthesia and the severity of ARF was assessed 24 h later by determining BUN and plasma creatinine concentrations.

Plasma catalytic Fe concentrations during the initiation phase of MH-ARF. To assess whether plasma, as well as urinary catalytic Fe activity rises after glycerol injection, five rats were anesthetized, a carotid artery catheter was placed, and a 0.5-ml baseline blood sample was obtained. Then the rats were injected with glycerol and repeat 0.5-ml blood samples were obtained 15 min and 2 h later. These samples (0, 15, and 120 min) were saved for catalytic Fe assay (see below). To determine whether DFO chelates a plasma catalytic Fe increment, five additional rats were treated as above, except they were subjected to the DFO/5% dextrose infusion (as previously discussed).

The following experiment assessed whether plasma catalytic Fe is cleared by the kidney, thereby contributing to urinary catalytic Fe activity. Three rats underwent bilateral nephrectomy through a midline abdominal incision and then they were injected with glycerol, as noted above. 2 h later, a plasma sample was obtained and analyzed for catalytic Fe. Renal catalytic Fe clearance was indirectly gauged by determining whether these 2-h plasma catalytic Fe concentrations were significantly higher than those observed in three additional rats subjected to sham bilateral nephrectomy and glycerol injection.

Intrarenal $^1$OH radical production during the initiation phase of MH-ARF. To assess whether plasma/urinary catalytic Fe increments cause excess intrarenal $^1$OH generation, the salicylate trap method was used (11–16). In brief, Na salicylate serves as a stable $^1$OH trap, 2,3- and 2,5-dihydroxybenzoic acids (DHBA) being byproducts of this reaction. Thus, increments in DHBA concentrations serve as indices of $^1$OH generation. Alternatively, decrements in DHBA indicate decreased $^1$OH production or $^1$OH scavenging. Supporting the utility of this technique are that (a) increased DHBA production occurs with $^1$OH generating systems (11–16); and (b) selected antioxidants (oxypurinol, dimethyliourea, DFO) decrease DHBA production both in vivo and in vitro (11, 16, and unpublished observations by this laboratory). Eight rats were anesthetized, they were injected with Na salicylate, 100 mg/kg i.p., and then half were injected with glycerol, the remaining half serving as controls. 2 h later, the kidneys were resected, cooled to 4°C, the cortical/outer medullary regions resected, homogenized, and extracted in 4 vol of 10% trichloroacetic acid, and then subjected to high performance liquid chromatography (System B) (16), the elutions being conducted with citrate/acetate buffer (pH 2.3/34% methanol). The 2.3-, 2,5-DHBA, and salicylate concentrations were quantified by electrochemical detection (15, 16) (sensitivity, 0.1 nmol/ml). The DHBA results were expressed in two ways: (a) as absolute concentrations per gram tissue dry wt; and (b) as absolute concentrations divided by the salicylate concentration (thereby factoring for differences in DHBA precursor accumulation within each kidney) (16).

In vitro experiments: effects of DFO and mannitol therapy on Fe-mediated proximal tubular segment cytotoxicity. Fe in molar excess. The following experiment was performed to assess whether mannitol and DFO confer direct additive/synergistic protection against Fe-mediated proximal tubular cell injury and whether such protection correlates with decrements in $^1$OH levels. To this end, proximal tubular segments (PTS) were harvested from normal rats by a previously described technique (17). In brief, the rats were subjected to retrograde aortic perfusion with a balanced salt solution to render the kidneys bloodless (17), they were resected, and the cortical tissues were isolated and minced with a razor blade at 4°C. The resulting tissues were digested with a collagenase containing buffer to which 1 mM DFO was added to chelate any free Fe generated during the mincing and digestion process (16). Viable PTS were recovered using a 31% Percoll gradient (17), and then they were washed 5× to remove any remaining collagenase and DFO. The PTS were suspended to a concentration of 2–4 mg PTS protein/ml in a buffer consisting of NaCl 100 mM, KCl 2.1 mM, NaHCO3, 25 mM, KH2PO4, 2.4 mM, CaCl2 1.2 mM, MgCl2 1.2 mM, MgSO4, 1.2 mM, glucose 5 mM, Na lactate 4 mM, Na butyrate 10 mM, alanine 1 mM, Na salicylate 1 mM and dextran T40 0.6%; gassed with 95% O2/5% CO2; final pH 7.40. The PTS preparations ($n = 4$; each one using cortices from two rats) were warmed to 4°C (isolation temperature) to 36°C over 15 min. As an assessment of PTS viability, a 150-μl aliquot was used to determine baseline percentage of LDH released (percentage of the total LDH in the PTS supernatant) (17), and then each preparation was divided into eight equal aliquots as denoted below:
(a) Fe challenge/no protective agents. This aliquot was incubated with 5 mM FeSO₄ as the oxidant challenge.
(b) Fe challenge + DFO addition. This Fe challenged (5 mM FeSO₄) aliquot had a molar deficit of DFO (4 mM) added such that complete cytoprotection would not result.
(c) Fe challenge + DFO + mannitol addition. This aliquot was identical to aliquot No. 2 except that 100 mM mannitol was added to determine whether it would improve upon the DFO-mediated protection.
(d) Fe challenge + mannitol addition. To ascertain whether mannitol exerted an independent cytoprotective effect in aliquot No. 3, aliquot No. 4 was treated with 5 mM FeSO₄ + 100 mM mannitol (11) without DFO.
(e) DFO alone/no Fe addition. This non-Fe exposed PTS aliquot was incubated with 4 mM DFO alone to ascertain its independent effect.
(f) DFO + mannitol/no Fe addition. This PTS aliquot was incubated with 4 mM DFO plus 100 mM mannitol to determine their combined effect in the absence of added FeSO₄.
(g) Mannitol alone/no Fe addition. 100 mM mannitol was added in the absence of DFO or Fe to determine its independent effect.
(h) PTS with only buffer addition. This aliquot represented a control incubation.

Each of the above incubations were maintained at 36°C for 45 min in a shaking water bath. At the end of the incubation, PTS cytotoxicity was determined by reassessing the percentage of LDH released. The degree of "OH generation/scavenging was assessed by the salicylate trap method, as noted above. Thus, after completion of the above incubations, PTS aliquots (100 μl) were extracted in 4 vol of 10% TCA, centrifuged for 2 min, and the supernatants were added to the citrate/acetate buffer and analyzed for 2,3- and 2,5-DHBA (11, 16). Special care was taken to assure that DFO (and any resulting ferroxamine), did not coelute with the DHBA peaks, thereby artifactually raising DHBA concentrations.

DFO and mannitol effects on in vitro Fe-mediated cytotoxicity: DFO in molar excess. In this experiment, the effects of DFO±mannitol on in vitro Fe toxicity was assessed when the DFO was present in slight molar excess of the total amount of Fe added (6 mM DFO and 5 mM Fe). In all other respects, this experiment was repeated exactly as described above (n = 4 PTS preparations).

Effect of the Fe/DFO molar ratio on DFO-mediated cytoprotection and "OH generation. In the above in vitro experiments, the different Fe/DFO molar ratios employed (5:4 or 5:6) produced different degrees of LDH release and "OH (DHBA) generation. The present experiment more fully explored these relationships. Three separate PTS preparations were each divided into seven equal aliquots as follows: (a) 5 mM Fe/no DFO addition; (b) 5 mM Fe/2 mM DFO; (c) 5 mM Fe/4 mM DFO; (d) 5 mM Fe/6 mM DFO; (e) 5 mM Fe/8 mM DFO; (f) 5 mM Fe/no DFO; and (g) 8 mM DFO/no Fe. After 45 min of incubation, the percentage of LDH released and DHBA concentrations were assessed.

Bovine serum albumin oxidation. To validate that the salicylate trap method accurately reflects Fe/DFO oxidant effects, the following experiment was performed: Bovine serum albumin ([BSA] 2.5 mg/ml H₂O) was incubated with (a) no addition; (b) FeSO₄, 1 mM; (c) DFO, 1.5 mM; (d) FeSO₄ + DFO; (e) FeCl₃, 1 mM; (f) FeCl₃ + DFO; (g) H₂O₂, 10 mM; and (h) H₂O₂ + DFO (n = 5–6 each). After a 1-h 37°C incubation, BSA’s carbonyl content, an index of protein oxidation (18, 19) was assessed by the method of Stadtman (19). To determine whether FeSO₄/DFO-mediated protein oxidation was "OH dependent, aliquot No. 4 was repeated 3× in the presence of 100 mM mannitol.

H₂O₂ mediated PTS injury: DFO and mannitol effects on LDH release and "OH generation. In the above described PTS experiments, Fe was the imposed oxidant challenge. However, data from Guidet and Shah (20) indicate that in vivo MH-ARF (gynecologic model), excess H₂O₂ generation occurs. Thus, the present experiment evaluated (a) whether H₂O₂-mediated cytotoxicity can be blunted with mannitol and DFO therapy; (b) whether H₂O₂ addition leads to excess PTS "OH generation (e.g., via the Haber Weiss reaction); and (c) whether DFO and mannitol influence these processes. To these ends, the PTS preparation procedure was modified in order to eliminate DFO from the PTS isolation process. This was done because it is theoretically possible that DFO, when added to the collagenase buffer, could have rendered the PTS Fe-deficient, thereby inhibiting the ability of H₂O₂ to generate "OH via the Haber Weiss reaction. Thus, PTS were prepared with two modifications: (a) DFO was not added to the collagenase digestion buffer; and (b) the kidneys were not perfused in vivo; thus, the mining/digestion process occurred in the presence of intrarenal blood, leaving transferrin to bind free Fe generated during collagenase digestion. Five PTS preparations were so isolated, each one being divided into six aliquots: (a) control incubation (no addition); (b) 15 mM H₂O₂ addition (shown in preliminary experiments to be the minimal dose necessary to induce consistent cytotoxicity (LDH release); (c) 15 mM H₂O₂ + 4 mM DFO; (d) 15 mM H₂O₂ + 100 mM mannitol; (e) 15 mM H₂O₂ + 4 mM DFO + 100 mM mannitol; and (f) 15 mM H₂O₂ + 25 mM dimethylthiourea ([DMTU]), a dose at least equipotent to 100 mM mannitol as an "OH scavenger) (11). After 45 min, the percentage of LDH release and DHBA concentrations were determined. To prove that 25 mM DMTU had no direct cytotoxic effect, potentially masking a beneficial action against H₂O₂, six additional PTS aliquots had LDH release determined in the presence or absence of 25 mM DMTU (n = 3 each).

Influence of H₂O₂ addition on PTS catalytic Fe activity. The following experiment assessed whether H₂O₂ increases PTS catalytic Fe activity, suggesting a possible link between H₂O₂ and Fe-mediated PTS toxicity (other than "OH production via the Haber Weiss reaction). Four PTS preparations, isolated without DFO addition, were each divided into four aliquots: two were incubated with 15 mM H₂O₂, while two were incubated with only an equal amount of buffer addition. After 20 min (a time before substantial cell killing occurs), the percentage of LDH release was determined, and each PTS suspension was assayed for catalytic Fe activity, as described below.

Bleomycin assay for catalytic Fe activity. Catalytic Fe was measured in the above experiments by a modification of the bleomycin method of Gutteridge and Hou (21). All measurements were made in duplicate. The reagents, unless otherwise stated, were from Sigma Chemical Co. (St. Louis, MO) and were dissolved in deionized water ([DW], Milli-Q) pretreated with Chelex-100 to remove contaminating Fe. Reagent solutions were subsequently treated with Chelex-100 and stored in plastic containers. Plastic incubation tubes contained, in order: 0.5 ml of 1 mg/ml DNA (type 1, calf thymus); 0.1 ml of 0.1 U/ml bleomycin sulfate; 0.1 ml of 50 mM MgCl₂; 0.1 ml of 1.0 M imidazole, pH 7.3; 0.1 ml of sample; and 0.1 ml of 8 mM ascorbic acid (ACS reagent grade). Samples were incubated by shaking for 2 h, at 37°C. The reactions were stopped with 0.1 ml of 1.0 M EDTA addition (Na salt, pH 7.3) and developed with 1.0 ml of 1% (wt/vol) thiobarbituric acid in 50 mM NaOH and 1.0 ml of 25% (vol/vol) HCl. The samples were heated for 10 min at 100°C and then cooled. 1.5 ml of n-butanol was added and mixed, and the mixture was centrifuged for 15 min at 1,100 g, room temperature. A₅₅₀ of the supernatant was measured vs n-butanol. Catalytic Fe concentrations were calculated by interpolation on a standard curve generated with FeCl₃ standards (0–10 μM) in Chelex-treated DW. Plasma and urine samples were diluted 1:10 before assay. (Urine samples obtained from five anesthetized normal rats served to establish normal catalytic Fe concentrations.)

The PTS suspension was assayed for catalytic Fe, as noted above (the cells being lysed in the reaction mixture). Given the low Fe concentrations, they were assayed undiluted. (It was first demonstrated that any remaining H₂O₂ did not artifactualy raise the Fe concentrations, as determined under the assay conditions used.)

Calculations and statistics. All values are given as means ± 1 SEM. The in vivo data were analyzed by either unpaired Student’s t test (two groups) or by one-way analysis of variance (ANOVA) with aftertesting by unpaired t test with Bonferroni corrections for multiple comparisons. The PTS data were first analyzed by ANOVA, followed by paired Student’s t test with Bonferroni corrections as aftertests. In the in vivo
experiments, the 2,3- and 2,5-DHBA concentrations were analyzed separately because of suggestions that isolated hepatic microsomal P450 may form 2,5-DHBA independent of \(^*\)OH (13). (However, data from this laboratory indicates that in vivo renal 2,5-DHBA production is in fact \(^*\)OH dependent) (16). In PTS suspensions, work from this laboratory (e.g., 11, 16) indicates that 2,5- and 2,3-DHBA each equally reflect \(^*\)OH production (e.g., parallel increases with \(^*\)OH-generating systems; parallel decrements with \(^*\)OH scavengers; slow equivalent percentage of increments in 2,3- and 2,5-DHBA production during control incubations; dissociation of 2,5-DHBA production from nonoxidant cell injury). Thus, in the PTS experiments, total \(^*\)OH production was gauged by the sum of the 2,3- and 2,5-DHBA concentrations. The histologic material was analyzed as follows: 5 \(\mu\m\) paraffin-embedded sections were stained with hematoxylin and cosin and the extent of proximal tubular necrosis was graded in a blinded fashion as: mild (1*), moderate (2*), or severe (3*) (\(\sim <10\%, 10-30\%, \text{and } 30-50\%\) of PFS showing evidence of necrosis). These scores were compared by Wilcoxon Rank sum test. Although distal heme protein cast formation was also apparent, its extent was considered too limited to permit semi-quantitative analysis (see below). Statistical significance throughout the study was judged by a \(P\) value of < 0.05.

**Results**

*In vivo experiments: severity of MH-ARF* (Fig. 1). Mannitol infusion in the absence of DFO caused a marked diuresis, averaging 8.3±1.4 ml over the 0–2-h post-glycerol injection period. The addition of DFO to the mannitol infusion did not significantly affect this urine flow rate (7.0±1.2 ml/2 h). By unpaired Student’s \(t\) test analysis, the DFO + mannitol treated rats had significantly better renal function than the mannitol only group, as assessed by both the 24-h BUN (\(P < 0.01\)) and plasma creatinine concentrations (\(P < 0.005\); see Fig. 1). The degree of heme pigment cast formation 24 h post-glycerol injection was mild in both groups (\(<10\%\) of distal tubular segments involved), and the extent of this finding did not appear to be affected by the DFO therapy. However, the extent of proximal tubular necrosis was significantly less in the mannitol + DFO vs the mannitol only group (score 1.3±0.15 vs 2.6±0.16; \(P < 0.01\)).

DFO therapy in the absence of mannitol infusion appeared to decrease the degree of azotemia, compared to the untreated glycerol group, but the BUN and creatinine values for these two groups did not statistically differ (Fig. 1). The DFO group had twice the 0–2 h urine flow rate as the untreated glycerol controls (1.9±0.2 vs 0.9±0.2 ml, respectively; \(P < 0.015\)), indicating that DFO exerted a modest diuretic effect.

An overall comparison of all four groups of rats (by one-way ANOVA followed by unpaired Student’s \(t\) test with Bonferroni corrections as aftertests) revealed that the combined DFO + mannitol group had significantly lower BUN and creatinine concentrations than any of the other groups. Mannitol in the absence of DFO also conferred significant protection, compared to the glycerol only group.

*Urine and plasma Fe concentrations.* Normal rat urine had no detectable catalytic Fe. Glycerol injection caused a marked increase in urinary catalytic Fe concentrations (33±2 \(\mu\m\)), yielding a 0–2 h postglycerol urinary output of 31±8 nmol. Mannitol treatment halved the urinary catalytic Fe concentrations (16±0.5 \(\mu\m\); \(P < 0.01\) compared to glycerol only group), presumably by dilution, and the diuresis induced a fourfold increase in the amount excreted (128±23 nmol; \(P < 0.002\) vs glycerol controls, 31±8 nmol). Infusion of DFO, either with or without mannitol, reduced the catalytic Fe content to virtually undetectable levels (\(<1\mu\m\) in every rat tested).

Normal rat plasma had no bleomycin-detectable Fe. However, by 15 min and 2 h post-glycerol injection, marked catalytic Fe increments were detected (23±4 and 34±4 \(\mu\m\), respectively), quantitatively approximating those in urine. However, unlike urine, DFO treatment did not eliminate the plasma catalytic Fe activity (15 min: 24±2 \(\mu\m\); 2 h: 25±3 \(\mu\m\); NS vs no DFO treatment). Bilateral nephrectomy before glycerol injection significantly increased the plasma catalytic Fe concentration (47±4 \(\mu\m\) vs 28±4 \(\mu\m\) for sham nephrectomy controls; \(P < 0.03\)), indicating that (a) the kidney is not required for production of plasma catalytic Fe, and (b) the urine is a route for its elimination.

*Intrarenal DHBA concentrations.* Neither 2,3- nor 2,5-DHBA concentrations (nmol/gm dry wt) were elevated in the glycerol rats, compared to their nonglycerol treated counterparts (glycerol: 2,3- and 2,5-DHBA, 2.0±0.2; 19±3; controls: 2.0±0.4, 18±5, respectively). Factoring these values by the intrarenal salicylate concentration still revealed comparable degrees of DHBA production (glycerol: 2,3- and 2,5-DHBA, 2.1±0.3, 21±5 pmol/nmol salicylate; controls: 2.3±0.1, 19±2, respectively). (Of note, in pilot studies performed in an identically fashioned, except for using a 7 ml/kg rather than a 10 ml/kg glycerol challenge, a lack of excess 2,3- and 2,5-DHBA generation was also apparent, compared to controls.)

*PTS experiments:* 5 \(\mu\m\) Fe/4 \(\mu\m\) DFO + mannitol addition (Fig. 2). The percentage of LDH released after 15 min of rewarming and after 45 min of incubation under control conditions were 6±1% and 11±0%, respectively (typical for all of the PTS experiments). Neither mannitol nor DFO, either alone or in combination, altered LDH release in the absence of the Fe challenge (Fig. 2C). 5 \(\mu\m\) Fe addition caused marked cytotoxicity (39±1% LDH release), a result which, surprisingly, was not significantly affected by 4 \(\mu\m\) DFO or mannitol addition, either alone or in combination (Fig. 2D).

The PTS groups incubated in the absence of the exogenous Fe challenge had DHBA concentrations ranging from 1.4–2.7 nmol DHBA/ml PTS buffer (means presented in Fig. 2A). Approximately 95% of the totals were 2,5-DHBA. Fe addition alone caused no significant total DHBA increment (2.3±0.2
Figure 2. LDH release and DHBA concentrations from PTS in the absence of Fe addition (A, C) or in the presence of 5 mM Fe (B, D)±antioxidant therapy [100 mM mannitol, 4 mM DFO, or an antioxidant (3)]. Neither DFO nor mannitol significantly altered LDH release or DHBA concentrations in the absence of Fe. Fe caused significant cytotoxicity (LDH release), despite no increment in DHBA production. DFO conferred no cytoprotection against the Fe challenge and paradoxically increased DHBA production (P < 0.01) when coincubated with Fe. Mannitol scavenged the Fe-DFO-mediated DHBA increment, despite conferring no cytoprotection, and it also lowered DHBA in the presence of Fe without DFO.

Figure 3. LDH release and DHBA concentrations in the absence of Fe addition (A, C) or in the presence of 5 mM Fe (B, D)±antioxidant therapy (100 mM mannitol; 6 mM DFO; O, no antioxidant). These results were similar to those found in the Fig. 2 experiments, except that DFO conferred some cytoprotection against Fe toxicity (P < 0.05) and that the Fe/6 mM DFO-mediated DHBA increment was higher than that seen with Fe/4 mM DFO. Mannitol still had no protective effect despite scavenging the Fe-DFO generated *OH increment.

Figure 4. LDH release and DHBA concentrations following exposure to different Fe/DFO molar ratios. DFO conferred progressive cytoprotection against the 5 mM Fe challenge (DFO concentrations vs LDH release; r = −0.93). This cytoprotection correlated with a progressive increase in DHBA production (r = 0.96; DFO dosage vs log10 DHBA concentrations). Neither DFO alone nor Fe alone significantly increased DHBA production.

**nmol/ml** (Fig. 2 B). However, when 4 mM DFO + 5 mM Fe were added, both 2,3- and 2,5-DHBA concentrations rose in every instance (total, 3.3±0.2 nmol/ml; P < 0.01 vs Fe incubation alone). Mannitol addition decreased 2,3- and 2,5-DHBA production in the presence of Fe (total DHBA: Fe, 2.3±0.2; Fe + mannitol, 1.3±0.2 nmol/ml; P < 0.01) despite the fact that no cytoprotection resulted. Similarly, mannitol eliminated the DHBA increment that resulted from Fe + DFO addition (Fe + DFO, 3.3±0.2; Fe + DFO + mannitol, 1.8±0.2 nmol/ml; P < 0.01), again without inducing cytoprotection.

**PTS experiments: 5 mM Fe/6 mM DFO plus mannitol addition (Fig. 3).** As in the above experiments, neither mannitol nor DFO addition altered LDH release in the absence of exogenous Fe (Fig. 3 C). Once again, Fe caused 39±2% LDH release. However, in contrast to 4 mM DFO, 6 mM DFO induced significant protection, decreasing the percentage of LDH released to 24±3% (P < 0.05; Fig. 3 D). Mannitol exerted no protective influence, either in the presence or absence of concomitant DFO therapy.

As noted above, neither mannitol, DFO, nor Fe, when added by themselves to PTS, caused a significant change in total DHBA concentrations (Fig. 3 A, B). However, Fe + 6 mM DFO more than doubled the total DHBA levels (P < 0.01; Fig. 3 B), again because of increments in both 2,3- and 2,5-DHBA. Mannitol significantly lowered these DHBA concentrations either when it was coincubated with Fe (mannitol + Fe, 1.4±0.1; Fe alone, 2.6±0.1 nmol/ml; P < 0.01), or in the presence of Fe + DFO (Fe + DFO, 5.3±0.3; Fe + DFO + mannitol, 3.1±0.3 nmol/ml; P < 0.01). However, despite these reductions, no cytoprotection resulted, as noted above.

**PTS experiments: 5 mM Fe and 0–8 mM DFO, effects on *OH production and cytotoxicity (Fig. 4).** As depicted in Fig. 4, there was a striking inverse correlation between the amount of DFO added to the 5 mM Fe challenge and the extent of LDH released (2, 4, 6, and 8 mM DFO → 38%, 31%, 20%, and 12% LDH release, respectively; r = −0.93). Only the 8 mM DFO dose was sufficient to completely prevent the Fe-mediated cytotoxicity (no Fe addition: 10±1% LDH release; 8 mM DFO + 5 mM Fe: 12±2% LDH release, NS). A correlate of this cytoprotection was a progressive, paradoxical increase in *OH generation, as assessed by both 2,3- and 2,5-DHBA production (5 mM Fe + 2, 4, 6, or 8 mM DFO: 2.3, 3.0, 5.3, and 12.0 nmol total DHBA/ml, respectively; r = 0.96, using DFO dose and log10 DHBA concentrations). Of note, this finding could not be attributed to DFO alone since 8 mM DFO, added in the absence of the Fe challenge, caused no DHBA generation (Fig. 4, top).

**BSA oxidation (Fig. 5).** FeSO4, FeCl3, and H2O2 each induced a modest increase in the BSA carbonyl content (> 95% confidence band for BSA alone; see Fig. 5, shaded area). DFO
had no independent effect on BSA carbonyl content. However, in the presence of FeSO₄, DFO caused a marked carbonyl content increment which could be totally blocked by mannitol addition. In contrast, FeCl₃-mediated BSA oxidation was decreased, not increased, by DFO. DFO had no significant effect on the BSA oxidation induced by H₂O₂ addition.

PTS experiments: H₂O₂ challenge and evaluation of protective agents (Fig. 6). After 45 min of incubation, H₂O₂ induced significant cytotoxicity (30±2% LDH release vs 13±1% for control incubation; *p* < 0.01) (Fig. 6). Addition of DFO completely abolished this H₂O₂-mediated damage (12±1% LDH release; NS vs controls). However, neither mannitol nor DMTU addition conferred cytoprotection (27±2% and 33±1% LDH release, respectively; NS vs H₂O₂ alone). DMTU in the absence of H₂O₂ had no impact on PTS viability (DMTU and control incubation, each 13±1% LDH release). The addition of H₂O₂ did not cause an increase in either 2,3- or 2,5-DHBA generation: rather, H₂O₂ tended to lower the total DHBA levels (control values, 1.1 nmol/ml; H₂O₂, 0.8 nmol/ml; *p* < 0.05, but NS after Bonferroni correction). No other significant differences in DHBA concentrations were apparent in these H₂O₂ experiments.

After 20 min of control incubation, the Fe concentrations in the PTS suspension were 2.08±0.19 μM. The paired PTS aliquots incubated for 20 min with H₂O₂ developed higher Fe concentrations in every instance (3.39±0.30 μM; *p* < 0.003). Thus, H₂O₂ addition raised the PTS catalytic Fe content by 63%. The percentage of LDH released in these 20 min experiments were: controls, 14±1%; H₂O₂, 20±1%, respectively (*p* < 0.005).

**Discussion**

Mannitol is generally considered a mainstay for prophylaxis of MH-ARF. However, in a recent study, this laboratory found that when mannitol is used in the glycerol ARF model, its protective influence is incomplete, mitigating azotemia and heme protein cast formation, but not the extent of proximal tubular necrosis (4). In part, mannitol’s inability to confer more complete functional and cellular protection may relate to the fact that during myoglobinuria, an active mannitol diuresis exacerbates tubular cell ATP depletion, probably because of an abrupt increase in GFR, and hence, in tubular metabolic work (4). Because of these therapeutic limitations, the present study has sought to determine whether DFO addition can improve upon mannitol’s overall protective effects.

As depicted in Fig. 1, when DFO was added to the mannitol infusion, the extent of azotemia was almost halved, indicating an approximate doubling of GFR. In addition, DFO was able to decrease the extent of proximal tubular necrosis; thus, unlike mannitol (4), it confers a cytoprotective effect. A pertinent question is whether DFO yields this additive protection by further stimulating a diuresis, and hence, heme protein excretion during the initiation phase of the renal injury (e.g., 0–2 h post-glycerol injection). This possibility is suggested by the fact that DFO doubled the 0–2 h urinary output in the absence of mannitol infusion. However, this hypothesis seems untenable for two reasons: (a) under conditions of combined DFO + mannitol infusion, the urine output was, if anything, slightly lower in the combined therapy group, compared to mannitol treatment alone; and (b) in neither the mannitol nor the combined treatment group did the degree of protection correlate with the 0–2 h urinary outputs (e.g., BUN, creatinine vs urine output, *r* = −0.1 to +0.1; range of urine outputs, 2–16 ml/2 h). This latter observation indicates that although a diuresis confers protection, there is a maximal degree of benefit that can be achieved, so that further stimulation of urine output does not confer incremental benefits. The present in vivo experiments also serve to contrast relative degrees of protection afforded by mannitol vs DFO therapy. As used, mannitol alone (but not DFO alone) caused statistically significant improvements in renal function. Thus, these results suggest that were DFO to undergo future clinical trials for prevention of MH-ARF, it should not be tested as a substitute for mannitol, but rather, as an adjunctive therapy.

Having defined DFO’s protective potential in the above experiments, the second goal of this study was to test previously unproven assumptions that (a) MH-ARF is associated with catalytic Fe generation, capable of mediating free radical reactions; and (b) that DFO can chelate this catalytic Fe burden.
To these ends, urinary catalytic Fe activity was sought during the initiation phase of the glycerol ARF model. Normal urine had no detectable catalytic Fe, as assessed by the bleomycin assay. However, after glycerol injection, a striking increment resulted. Both mannitol and DFO markedly influenced these urinary catalytic Fe concentrations: mannitol approximately halved them (probably because of dilution), while inducing a fourfold increase in Fe excretion. That this latter result strongly correlated with the 0–2 h urinary output (r = 0.93) suggests its dependence on the urinary flow rate. In contrast, DFO, either in the presence or absence of mannitol infusion, essentially eliminated urinary catalytic Fe activity, undoubtedly caused by chelation. Thus, one could speculate that DFO improves upon mannitol-mediated protection by removing the renal catalytic Fe burden, not achievable with diuresis alone. However, this remains only an assumption since the critical issue is whether DFO also eliminates the tubular cell catalytic Fe load. Unfortunately, this cannot be accurately gauged in in vivo experiments since high intraluminal catalytic Fe and DFO concentrations contaminate tissue homogenates.

Although it is generally assumed that a renal catalytic Fe burden during myohemoglobinuria results from intrarenal heme protein Fe release; e.g., during lysosomal protein degradation, the present study strongly suggests that it may also, in large part, be derived from the circulation. Within 15 min of glycerol injection, striking plasma catalytic Fe activity had been generated, its concentration continuing to increase during the next 105 min. That bilateral nephrectomy plus glycerol injection caused higher plasma catalytic Fe levels than glycerol injection alone indicates that (a) this circulating catalytic Fe is generated at extrarenal locations; and (b) the kidney is a significant route for its elimination, presumably by glomerular filtration. Thus, the proximal tubular catalytic Fe burden could potentially originate from an intraluminal, not an intratubular cell location; e.g., lysosomes. Interestingly, DFO had no significant impact on the plasma catalytic Fe activity, in marked contrast to urine. The reason for this is unknown, although rapid urinary DFO excretion, and hence plasma elimination, is one possibility. One potential consequence of a high, circulating catalytic Fe load could be direct vascular and/or extrarenal tissue injury.

Because DFO and mannitol can potentially influence MHA-ARF by intravascular/intraluminal, as well as by direct cellular effects, the influence of DFO+mannitol therapy on direct Fe-mediated PTS cytotoxicity was assessed. Because previous work from this laboratory has indicated that 1–2 mM Fe is needed to induce cytotoxicity in this system (11), and because additive/synergistic protection was sought, an even larger (5 mM) Fe challenge was chosen for these experiments. In light of the above in vivo data indicating that μM, not mM, Fe concentrations are present during myohemoglobinuria, a compelling question is whether sufficient catalytic Fe is generated in vivo to induce direct proximal tubular cytotoxicity, or whether the in vivo catalytic Fe activity merely represents an epiphenomenon. However, because the intracellular Fe concentrations and sites of accumulation which occur in vivo and in vitro are unknown, this remains an important but unanswered question. Nevertheless, the present in vitro studies can provide some insights as to whether mannitol and DFO exert additive protective effects against a catalytic Fe challenge, and whether that protection correlates with decreased OH production or activity.

To initially address these issues, PTS were incubated with 5 mM Fe2⁺ molar deficit of DFO (4 mM), so that incomplete cytoprotection would result. Thus, if mannitol and DFO were to confer additive benefits, they should have been apparent in this experiment. Surprisingly, 4 mM DFO conferred no protection whatsoever, indicating that it must be present in at least molar equivalence to Fe to have a beneficial impact. Similarly, mannitol exerted no protective influence (Fig. 2), either in the presence or absence of DFO therapy. To help substantiate these findings, experiments were conducted with DFO in slight molar excess (6 mM) to the Fe challenge (Fig. 3). Under these circumstances, DFO did confer protection, albeit incomplete, but once again, mannitol had no additive benefit. Thus, these two sets of experiments strongly suggest that DFO + mannitol do not induce additive protection in vivo by conferring a dual cytoprotective influence against catalytic Fe-mediated injury. Interestingly, Fe addition induced cytotoxicity without apparent excess OH(DHBA) generation, mannitol did not block Fe toxicity despite lowering DHBA to subnormal values (indicative of OH scavenging), and 6 mM DFO induced protection despite increasing OH production. Thus, these results, in concert with previous suggestions from this laboratory, indicate that Fe-mediated cytotoxicity and OH generation are not necessarily pathogenetically linked (11).

To further explore this possibility, the effects of different Fe/DFO ratios on OH production and Fe-mediated cytotoxicity were assessed. As depicted in Fig. 4, a striking dose-response relationship was noted: the higher the DFO/Fe ratio, the greater the degree of cytoprotection and the greater the OH(DHBA) production rate, again seemingly dissociating OH generation and cytotoxicity. DFO in the absence of Fe did not affect DHBA levels. Thus, these results strongly suggest that DFO stimulated OH generation by accelerating Fe²⁺ oxidation to Fe³⁺, the chelated form. To validate this hypothesis, the effects of DFO on Fe²⁺, Fe³⁺, and H₂O₂-mediated BSA carboxyl content was assessed. As depicted in Fig. 5, Fe²⁺-mediated BSA oxidation was dramatically increased by DFO, indicating the drug’s marked prooxidant effect. That mannitol prevented this Fe²⁺/DFO-induced BSA oxidation strongly suggests that it occurs by an OH dependent reaction. Finally, that DFO blocked Fe²⁺-induced BSA oxidation indicates that DFO’s prooxidant effect in the presence of Fe³⁺ is, in fact, dependent on its ability to accelerate Fe²⁺ oxidation. Thus, these BSA experiments strongly support the salicylate trap data, indicating that DFO’s cytoprotective effect can be expressed despite increased OH production. Given this conclusion, it is not at all surprising that even though mannitol can scavenge this DFO-generated OH excess (Fig. 2 B, 3B) cytoprotection does not result.

Because increased H₂O₂ production may occur during MHARF (20), and since DFO and OH scavengers may confer in vivo functional and morphologic protection (1–3), it has been hypothesized that heme pigment–derived Fe catalyzes OH production from H₂O₂ via the Haber Weiss reaction, thereby inducing tissue injury. Thus, another goal of this study was to test this hypothesis in vitro by studying whether H₂O₂ addition to PTS generates OH(DHBA) and whether DFO/OH scavengers blunt the resulting injury. To make certain that the PTS employed in these experiments were not Fe depleted, they were prepared without DFO addition to the collageenate digestion buffer and catalytic Fe content was confirmed by bleomycin assay. After 45 min of H₂O₂ incubation, modest
cytotoxicity was apparent, a result that was completely blocked by DFO addition to the incubation medium. Thus, these findings support previous observations obtained using cultured tubular cells (22, 23) and hepatocytes (24) that DFO blocks H₂O₂-mediated injury. However, the present data also suggest that this Fe-dependent H₂O₂ cytotoxicity is not ·OH-mediated for the following reasons: (a) H₂O₂ addition did not cause excess ·OH generation, as assessed by the salicylate trap method; and (b) mannitol did not confer protection. It could be argued that mannitol, a relatively impermeant solute, could not gain access to intracellular sites of ·OH production, and hence, a lack protection cannot be taken as evidence against ·OH-mediated injury. However, that DFO, a cell permeable ·OH scavenger, also did not protect supports the DHBA data, implying a non-·OH-dependent cytotoxic mechanism. Because H₂O₂ can mobilize Fe from proteins and/or labile intracellular pools (25, 26), the hypothesis was tested that H₂O₂ addition increases PTS catalytic Fe content, which then mediates toxicity. After 20 min of H₂O₂ addition, a 63% increase in PTS catalytic Fe activity was apparent. That this increase occurred before the onset of marked cytotoxicity suggests that the Fe increment could have been a mediator of the cell injury, rather than just a consequence of it. That DFO conferred complete protection against H₂O₂-induced injury further supports this assumption. Thus, it may be that H₂O₂ induces Fe-dependent injury not via ·OH formation, but rather, by generating catalytic Fe within PTS. Whether this liberated Fe induces direct toxicity, or whether H₂O₂ converts it to toxic byproducts (e.g., ferryl/perferryl ion) (27-29), remains to be defined. Whatever the exact mechanism, that mannitol did not affect H₂O₂ mediated injury further supports the assumption that its in vivo protective effect was via a nonoxidant dependent action.

Although the above in vitro experiments indicate that catalytic Fe and H₂O₂ may induce proximal tubular injury by a non- ·OH-dependent mechanism, the relevance of the PTS model to in vivo Fe/heme protein nephrotoxicity remains to be proven. For example, it is quite possible that in vivo/in vitro differences in catalytic Fe concentrations, routes of Fe delivery, intracellular sites of Fe accumulation, or functional/structural changes induced by the PTS isolation process render the above in vitro results nonapplicable to in vivo ARF. Furthermore, it is possible that salicylate, and oxidant challenges, delivered into the PTS fluid phase yield results not directly germane to intracellular events. Thus, the final goal of this study was to return to the in vivo MH-ARF model to further assess whether exaggerated ·OH production results, thereby supporting the possibility that DFO/mannitol therapy confers additive protection via anti-·OH effects. To this end, intrarenal ·OH generation was sought during the initiation phase of the glycerol ARF model, again using the salicylate trap method. However, no DHBA increments were observed, again suggesting that Fe/heme protein nephrotoxicity can occur without excess ·OH formation. Clearly, this experiment, by itself, does not conclusively exclude ·OH dependent renal injury during in vivo MH-ARF. However, when viewed in the context of (a) the above in vitro results which seemingly dissociate Fe/H₂O₂ cytotoxicity from ·OH generation; (b) recent in vivo data demonstrating that ·OH scavengers do not necessarily protect against MH-ARF (3, 4, 30); and (c) that lipid peroxidation may not result during MH-ARF (4, 30), also support this view. Thus, the critical pathways by which heme Fe/heme proteins induce tubular injury remain to be defined.

In conclusion, the present study indicates that (a) DFO and mannitol confer additive protection against the glycerol model of ARF. (b) Catalytic Fe is generated during the initiation phase of this model, gaining access to the systemic circulation and the kidney. (c) This circulating catalytic Fe load is, in part, cleared by the kidney, contributing to the intraluminal/tubular catalytic Fe burden. (d) DFO completely chelates urinary catalytic Fe activity, potentially eliminating its cytotoxic potential. However, that Fe-DFO binding can paradoxically drive ·OH production, and that MH-ARF may not generate excess ·OH, makes it critical not to equate DFO-mediated in vivo protection with an anti- ·OH effect. (e) Mannitol can both dilute urinary catalytic Fe activity and enhance its excretion. However, that mannitol does not (i) directly limit Fe/H₂O₂-mediated in vitro PTS injury; (ii) that it has limited access to intracellular locations; and (iii) that its in vivo protective influence correlates with decreased cast formation, not decreased cell necrosis (4), all suggest that its in vivo benefit is derived from a diuretic, not an ·OH scavenging effect. (f) H₂O₂-mediated PTS toxicity appears to be Fe- (but not ·OH-) dependent, since (i) H₂O₂ addition caused no apparent ·OH generation; (ii) ·OH scavengers (mannitol, DMTU) conferred no benefit; and (iii) H₂O₂ liberated catalytic Fe, the chelation of which caused complete protection. Thus, an enhancement of the intracellular catalytic Fe burden, rather than ·OH-mediated oxidant stress, may be the more critical determinant of H₂O₂-mediated tubular injury. However, the mechanism(s) by which Fe exerts this toxicity remains unknown.

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718 R. A. Zager