Kidney International, Vol. 66 (2004), pp. 144–156

Parenteral iron nephrotoxicity: Potential mechanisms and consequences[1]

RICHARD A. ZAGER, ALI C.M. JOHNSON, and SHERRY Y. HANSON

Department of Medicine, University of Washington, and the Fred Hutchinson Cancer Research Center, Seattle, Washington

Parenteral iron nephrotoxicity: Potential mechanisms and consequences.

Background. Parenteral iron administration is a mainstay of anemia management in renal disease patients. However, concerns of potential iron toxicity persist. Thus, this study was conducted to more fully gauge iron toxicologic profiles and potential determinants thereof.

Methods. Isolated mouse proximal tubule segments (PTS) or cultured proximal tubular [human kidney (HK-2)] cells were exposed to four representative iron preparations [iron sucrose (FeS), iron dextran (FeD), iron gluconate (FeG), or iron oligosaccharide (FeOS)] over a broad dosage range (0, 30 to 1000 μg iron/mL). Cell injury was assessed by lactate deyhdrogenase (LDH) release, adenosine triphosphate (ATP) reductions, cell cytochrome c efflux, and/or electron microscopy. In vivo toxicity (after 2 mg intravenous iron injections) was assessed by plasma/renal/cardiac lipid peroxidation [malondialdehyde (MDA)], renal ferritin (protein)/heme oxygenase-1 (HO-1) (mRNA) expression, electron microscopy, or postiron injection PTS susceptibility to attack.

Results. In each test, iron evoked in vitro toxicity, but up to 30× differences in severity (e.g., ATP declines) were observed (FeS > FeG > FeD = FeOS). The in vitro differences paralleled degrees of cell (HK-2) iron uptake. In vivo correlates of iron toxicity included variable increases in renal MDA, ferritin, and HO-1 mRNA levels. Again, these changes appeared to parallel in vivo (glomerular) iron uptake (seen with FeS and FeG, but not with FeD or FeOS). Iron also effected in vivo alterations in proximal tubule cell homeostasis, as reflected by the "downstream" emergence of tubule resistance to in vitro oxidant attack.

Conclusion. Parenteral iron formulations have potent, but highly variable, cytotoxic potentials which appear to parallel degrees of cell iron uptake (FeS > FeG ≫ FeD or FeOS). That in vitro injury can be expressed at clinically relevant iron concentrations, and that in vivo glomerular iron deposition/injury may result, suggest caution is warranted if these agents are to be administered to patients with active renal disease.

[1]**See Editorial by Alam et al, p. 457.**

Key words: iron dextran, iron sucrose, iron gluconate, oxidant stress, heme oxygenase 1.

Received for publication December 23, 2003
and in revised form January 29, 2004
Accepted for publication February 11, 2004

Administration of parenteral iron has become a mainstay for treating anemia in patients with end-stage renal disease (ESRD). This practice is required in order to offset dialysis-related blood (iron) loss, and the need to optimize hematopoietic responsiveness to exogenous erythropoeitin (Epo) therapy [1–3]. While generally regarded as safe, anaphylactic/oid reactions have been noted following intravenous iron injection, most commonly but not exclusively, with dextran preparations [4]. In addition to allergic reactions, each currently employed parenteral iron formulation [e.g., iron dextran (FeD), iron sucrose (FeS), and iron gluconate (FeG)] has the potential to induce oxidative stress [5–9]. For example, when administered intravenously, these agents may induce free radical generation [10] and lipid peroxidation [5], processes which can induce acute endothelial dysfunction (e.g., as denoted by perturbed forearm endothelial-dependent vasodilation) [10]. Additional support for the concept of iron-induced toxicity comes from a recent report [11] which indicates that clinically achievable concentrations of FeG or FeS can impair polymorphonuclear cell (PMN)/transendothelial migration. This could contribute to infectious complications in dialysis patients.

While the above evidence suggests potential acute toxicities, the long-term consequences of parenteral iron administration remain largely unknown. In this regard, it is noteworthy that iron-mediated oxidative stress can contribute to both atherogenesis [12–17] and chronic inflammation [18–22], each of which are leading causes of morbidity and mortality in ESRD patients [23–25]. Furthermore, because parenterally administered iron has direct glomerular, and as well as tubular access (via peritubular capillaries), it is conceivable that it might contribute to glomerular and/or tubulointerstitial disease progression [26–29]. That intravenous iron + Epo therapy is currently being administered to pre-ESRD patients underscores these concerns.

Given that parenteral iron therapy is likely to remain an integral component of renal disease patient management, it is imperative to better define its potential cytotoxic effects, and to ascertain whether different toxicity profiles exist amongst currently employed parenteral

iron formulations. Indeed, in a recent study performed in this laboratory using suprapharmacologic iron doses [5], a clear gradation of toxicity was apparent amongst four test agents [from most to least severe: FeS ≫ FeG ≫ FeD = Fe oligosaccharide (FeOS)]. However, the reason(s) for this differential in vitro toxicity, if it might be observed with more clinically relevant iron concentrations, whether in vivo toxicologic correlates exist, and the nature of underlying pathogenic mechanisms were not well defined. Hence, the present study was undertaken utilizing a number of experimental models (freshly isolated mouse proximal tubules, cultured human proximal tubular cells, and in vivo mouse experiments) to gain additional insights.

METHODS

Proximal tubule segment (PTS) experiments

Preparation of isolated mouse proximal tubules. Proximal tubules were isolated from normal CD-1 male mice (25 to 35 g) (Charles River, Wilmington, MA, USA), as previously described [30]. In brief, the mice were deeply anesthetized with pentobarbital (4 to 5 mg intraperitoneally), and the kidneys were resected through a midline abdominal incision. They were iced, the cortices were dissected, and the tissues were subjected to collagenase digestion. The tissues were passed through a stainless steel mesh, and then viable PTS were collected after pelleting through 32% Percoll [30, 31]. The recovered tubules were suspended in an experimentation buffer consisting of (in mmol/L): NaCl, 100; KCl, 2.1; $NaHCO_3$, 25; KH_2PO_4, 2.4; $MgSO_4$ 1.2; $MgCl_2$, 1.2; $CaCl_2$, 1.2; glucose, 5; alanine, 1; Na lactate, 4; Na butyrate, 10; 36 kD dextran, 0.6%; and gassed with 95% O_2/5% CO_2, pH 7.44). The final tubule protein concentration was ~2 to 4 mg/mL. Each PTS preparation was rewarmed to 37°C in a heated shaking water bath and divided into four to six equal aliquots (1.25 mL) in 10 mL Erlenmyer flasks, depending on the needs of individual experiments (see below).

Comparative effects of iron preparations on proximal tubule adenosine triphosphate dehydrogenase (ATP) content.

Dose response experiments. The purpose of this study was to compare dose-response toxicity effects of four test iron preparations. Given that previous studies demonstrated that mitochondrial dysfunction, as assessed by reductions in tubule ATP production, is a sensitive marker of iron toxicity [5, 31], tubule ATP concentrations, as well as lethal cell injury [% lactate dehydrogenase (LDH) release], were chosen as test biologic end points. Twelve individual sets of PTS were prepared, each was divided into five equal aliquots, and these were incubated ×30 minutes in a 37°C shaking water bath in the presence of 95% O_2/5% CO_2, under the following conditions: (*1*) control incubation; (*2*) 1000 μg/mL iron addition; (*3*) 500 μg/mL iron addition; (*4*) 250 μg/mL iron addition; and (*5*) 125 μg/mL iron addition. Each individual tubule preparation was used to test one of the four iron preparations: (1) FeS (Venofer) (American Regent, Shirley, NY, USA); (*2*) FeD (INFeD) (Watson Pharmaceuticals, Morristown, NJ, USA); (*3*) FeG (Ferrlecit) (Watson Pharmaceuticals); and (*4*) FeOS, an iron preparation currently in clinical trials (Pharmacosmos, Copenhagen, DK). In all, each of these preparations were tested in three separate dose-response experiments. After completing the 30-minute incubations, a sample of each aliquot was removed, adenine nucleotides were extracted in trichloroacetic acid, and then the samples were analyzed for ATP by high-performance liquid chromatography (HPLC) (nmol/mg tubule protein) [32]. An aliquot of each tubule suspension was also used to determine % LDH release.

pH control experiment. Because FeS stock solution has a pH of approximately 10.5, and because a high pH can induce cytotoxicity [33], a control for the above FeS additions was conducted with an equivalent amount of sucrose (300 mg/mL) with its pH adjusted to 10.5 by 1 N NaOH addition. Aliquots from four sets of tubules were incubated either under control conditions or with 62.5 μL of the alkaline sucrose solution (equivalent to the volume of the 1000 μg/mL FeS dosage). After 30-minute incubations, ATP concentrations and % LDH release were assessed.

Effects of low dose FeS and FeG on tubule ATP concentrations. The above dose-titration experiments indicated that FeS and FeG had the greatest suppressive effects on tubule ATP concentrations, with reductions being apparent at the lowest test concentration (125 μg iron/mL) (see **Results** section). The following experiment ascertained whether ATP reductions could be induced by even lower iron concentrations (i.e., within clinically achievable plasma iron concentrations). Four sets of tubules were prepared, each was divided into five equal aliquots, and incubated ×30 minutes as follows: group 1, control conditions; groups 2 and 3, with 30 or 60 μg/mL FeS iron; and groups 4 and 5, with 30 or 60 μg/mL FeG iron. ATP levels and LDH release were then assessed.

In vivo mouse experiments

Assessment of lipid peroxidation following intravenous iron treatment. The following experiments were undertaken to ascertain the relative degrees of lipid peroxidation induced by three representative test iron compounds: FeD, FeS, and FeG. These three compounds were selected because they manifested the greatest differential toxicity in the above described proximal tubule experiments (see **Results** section). Mice ($N = 18$) were placed in non-traumatic restraining cages, and they were injected via

Table 1. Mouse primers for quantitating heme oxygenase 1 (HO-1) mRNA in renal cortex

Genes	Primer sequences	Polymerase chain reaction conditions	Product size
Mouse GAPDH	5′-CTG CCA TTT GCA GTG GCA AAG TGG-3′ 5′-TTG TCA TGG ATG ACC TTG GCC AGG-3′	94°C – 45 sec; 57°C – 45 sec; 72°C – 45 sec; 28 cycles	437 bp
Mouse HO-1	5′-AAC ACA AAG ACC AGA GTC CCT CAC-3′ 5′-CAA GAG AAG AGA GCC AGG CAA GAT-3′	94°C – 45 sec; 57°C – 45 sec; 72°C – 45 sec; 28 cycles	288 bp

Primer sequences used for quantitating HO-1 mRNA in mouse renal cortex 4 hours following intravenous iron treatment (see text). Glyeraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified as a "housekeeping gene."

the tail vein with either 2 mg of iron ($N = 4$ of each of the above iron preparations), or with a sham tail vein saline injection ($N = 6$). The mice were then released from the restrainers and, 90 minutes later, they were deeply anesthetized with pentobarbital, as above. The abdominal cavities were opened, a plasma sample was obtained from the inferior vena cava, and then one kidney per animal was resected. The thorax was opened and the heart was removed. The tissues were placed on an iced plate. A piece of renal cortex and of cardiac apex were resected, the tissues rinsed in iced saline to remove contaminating blood, and then ~75 mg of renal cortex or heart tissue were homogenized in 1 mL of iced phosphate-buffered saline (PBS) containing 25 mmol/L desferrioxamine (DFO) to chelate any free iron which may have been generated during this process. Samples of tissue homogenates (200 μL) were then assayed for malondialdehyde (MDA) concentrations by the thiobarbituric acid method [34]. Tissue MDA concentrations were expressed as nmol/mg tissue protein. Plasma samples (200 μL), to which 25 mmol/L DFO was added, were also assayed for MDA with values being expressed as nmol/mL.

Parenteral iron effects on renal ferritin and heme oxygenase-1 (HO-1) expression. The following experiments were conducted to ascertain whether, and to what degree, the four test iron preparations impact renal cortical homeostasis, as assessed by the potential induction of ferritin and HO-1 proteins (redox-sensitive indicators). To this end, mice received every other day tail vein injections of 2 mg iron, administered as either FeS ($N = 5$), FeG ($N = 5$), INFeD ($N = 5$), or FeOS ($N = 4$). Each group of mice had their own simultaneous control groups which received equal volume tail vein saline injections. Approximately 24 hours following the last of the three injections, the mice were anesthetized with pentobarbital, the kidneys were removed, and the cortices were dissected on an iced plate.

Western blotting. The above noted renal cortical tissue samples were extracted for protein and probed by Western blot for ferritin and HO-1, using previously described general methodologies [35]. In the case of ferritin, 25 μg of protein extract were electrophoresed through a 12% Bis-Tris acrylamide Nupage gel (Invitrogen Life Technologies, Carlsbad, CA, USA) and probed with goat antiferritin antibody (catalog number SC-14416) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), according to manufacturer's instructions. For HO-1 detection, 50 μg of protein extract was electrophoresed as described above, and probed with rabbit anti-HO-1 antibody (catalog number SC-10789) (Santa Cruz Biotechnology) as the primary antibody as per manufacturer's instructions. Secondary detection of the anti-ferritin and anti-HO-1 antibodies was performed with either horseradish peroxidase (HRP)-labeled donkey antigoat IgG (catalog number SC-2020) (Santa Cruz Biotechnology) for ferritin or with HRP-labeled donkey antirabbit IgG (catalog number NA 934) (Amersham-Pharmacia, Piscataway, NJ, USA) for HO-1. Detection was by enhanced chemiluminescence (ECL Kit) (Amersham-Pharmacia). Western blot semiquantitative analysis was performed by band optical density scanning. Nonspecific secondary antibody staining was ruled out by the fact that the secondary antibody, in the absence of the primary antibody, did not identify the relevant protein bands (ferritin, ~25 kD; HO-1, ~32 kD). Equal protein loading/transfer was verified by India ink staining. A positive control consisted of renal cortical protein samples from mice 18 hours postinduction of glycerol induced-acute renal failure (which upregulates both HO-1 and ferritin) [36].

HO-1 mRNA expression following iron treatment. Stress induced changes in tissue ferritin concentrations are largely determined by posttranslational events, whereas HO-1 expression is regulated via oxidant stress-induced HO-1 gene transcription [37]. Therefore, to gain further insights into relative degrees of iron-induced oxidant stress, mice were injected with either FeD, FeG, FeS, or FeOS, as noted above ($N = 4$ to 6 per group). Controls consisted of ten mice subjected to tail vein saline injections. Four hours later, the mice were anesthetized with pentobarbital, and the kidneys resected. The renal cortical tissues were immediately placed into TRIzol reagent (Invitrogen Life Technologies) and total RNA was extracted according to the manufacturer's instructions. The final RNA pellet was brought up in RNase-free water to an approximate concentration of 3 mg/mL.

Reverse transcription (RT) and polymerase chain reaction (PCR) were performed using the 1st-Strand Synthesis Kit for RT-PCR (Ambion Inc., Austin, TX, USA), as previously described in detail [38]. The specific primers for HO-1 and glyceraldehyde-3-3-phosphate dehydrogenase (GAPDH) were designed with 50% to 60% GC composition (see Table 1). The similarity in annealing

temperature, but dissimilarity in PCR products, enabled a multiplexed reaction whose products were analyzed by agarose gel electrophoresis and ethidium bromide staining. cDNA bands were visualized and quantified by densitometry with a Typhoon 8600 scanner (Amersham Pharmacia Biotech). HO-1 cDNA bands were expressed as ratios to the simultaneously obtained GAPDH cDNA bands, the latter used as a housekeeping gene.

Renal histology. To assess whether parenteral iron treatment might induce structural renal alterations, mice which were subjected to the above intravenous iron treatment protocols (2 mg iron every other day × 1 week; $N = 2$ for each test agent) or to sham saline injections ($N = 3$). One day following the last injection, the kidneys were harvested, and prepared for either light or electron microscopy. For light microscopy, a midline slice of kidney (cortex to papilla) was fixed in 10% formalin and 4 μ paraffin-embedded sections prepared and stained with hematoxylin and eosin. For electron microscopy, 1 mm cubes of renal cortex were fixed by immersion in $^1/_2$ strength Karnovsky's fixative. Tissue sections were cut and evaluated by transmission electron microscopic analysis, as previously described [39]. At least four glomeruli from two different kidneys were extensively examined by electron microscopy.

Cultured proximal tubular [human kidney (HK-2)] cell experiments

Cytotoxicity and cellular loss of cytochrome c. The following experiment was undertaken to further ascertain relative degrees of iron-mediated cytotoxicity, as assessed by % LDH release and extracellular cytochrome c release (a marker of mitochondrial damage) [31]. To these ends, immortalized human proximal tubular (HK-2) cells were cultured in T-75 flasks with keratinocyte serum-free medium (K-SFM) and passaged by trypsinization every 5 to 6 days, as previously described [40]. For experimentation, the cells were seeded into 18 T-25 flasks. After an overnight postseeding recovery period, the cells were divided into six groups of three flasks each: (*1*) control cells ($N = 3$); (*2*) incubation with 100 μg/mL FeS iron; (*3*) incubation with 100 μg/mL FeG iron; (*4*) incubation with 100 μg/mL FeD iron; (*5*) incubation with 100 μg/mL FeOS iron; and (*6*) a second group of control incubated cells. The cells were maintained under routine culture conditions for 3 days. At the completion of the incubations, % LDH release was determined. Then, the cells which remained attached to the flasks were recovered by scraping with a cell scraper, and washed with Hanks' balanced salt solution (HBSS), and pelleted. The pellets were photographed with a digital camera. Then, cell protein extracts were prepared and probed for cytochrome c by Western blotting [31]. An equal amount of protein (8 μg) from each cell sample was applied.

Electron microscopic analysis of iron effects on HK-2 cell morphology. The following experiment was undertaken to ascertain the effect of the four test iron preparations on HK-2 cell morphology. To this end, a 6-well Costar plate was seeded with HK-2 cells and allowed to grow to near confluence. One well each was subjected to the following conditions: (*1*) control incubation; (*2*) incubation with 100 μg FeS iron; (*3*) incubation with 100 μg FeG iron; (*4*) 100 μg FeD iron; (*5*) 100 μg of FeOS iron; and (*6*) additional control culture. After an 18-hour incubation, the cell culture medium in each well was removed, and then a mixture of 1 part of $^1/_2$ strength Karnovsky's fixative/1 part fresh culture medium was added to the adherent cells. The cells were allowed to fix overnight. After dehydration and alcohol fixation, groups of cells were randomly lifted off the plates by applying small resin blocks to the monolayers. These blocks were then cut and processed for transmission electron microscopy, as previously described [41].

Combination in vivo/in vitro experiments

Intravenous iron injection with subsequent in vitro analysis of cytoresistance. A feature of acute sublethal renal tubular injury is the subsequent emergence of partial cell resistance to further attack [42–45]. In particular, iron-mediated injury induces resistance to further oxidative damage [36, 42]. Hence, the goal of this experiment was to ascertain whether parenteral iron administration can induce sublethal proximal tubular injury, and that this prior injury is denoted by the emergence of cytoresistance to subsequent iron-mediated tubular attack. To this end, four mice were injected with 2 mg of FeS via the tail vein (0.1 mL). Four mice subjected to equal saline tail vein injections served as controls. The mice were then provided with free food and water access (preliminary data indicated that no difference in food intake/body weight resulted from the iron injection). Eighteen hours postinjections, they were anesthetized with pentobarbital, the kidneys resected, and cortical proximal tubules were isolated, as above. The eight preparations (four postiron injection; four postsaline injection) were each divided into five equal tubule aliquots as follows: (*1*) control incubation (95% O_2/5% CO_2); (*2*) hypoxic incubation (95% N_2/5% CO_2); (*3*) exposure to 100 μmol/L antimycin A (a mitochondrial inhibitor); or (*4*) addition of 25 μmol/L ferrous ammonium sulfate (iron), complexed to the siderophore hydroxyquinoline (FeHQ), permitting iron to gain intracellular access [42]. After completing 15-minute incubations under each of these conditions, the extent of lethal cell injury was gauged by % LDH release. The results for the control and Venofer pretreatment groups were compared.

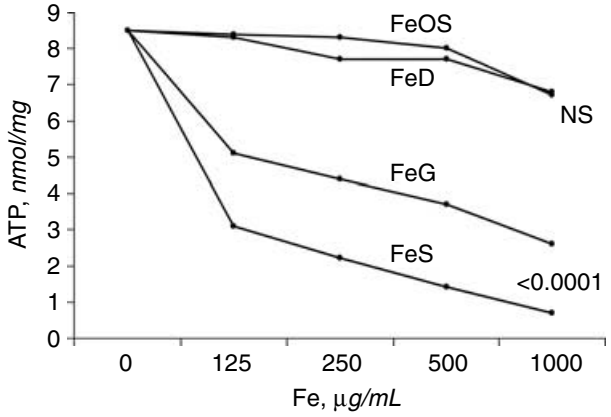

Fig. 1. Proximal tubular segment adenosine triphosphate (ATP) concentrations following 30-minute incubations with four test iron preparations: iron dextran (FeD), iron oligosaccharide (FeOS), iron gluconate (FeG), and iron sucrose (FeS). ATP concentrations are presented as nmol/mg tubule protein. FeD and FeOS caused only minimal ATP declines, and these were apparent at only the 1000 μg/mL iron concentration. In contrast, steep ATP declines were observed with both FeS and FeG, with the degree of ATP reductions being statistically greater with FeS vs. FeG ($P < 0.0001$; all dose-paired comparison). Standard error bars are not shown for clarity sake, but were all <0.4 nmol/mg protein.

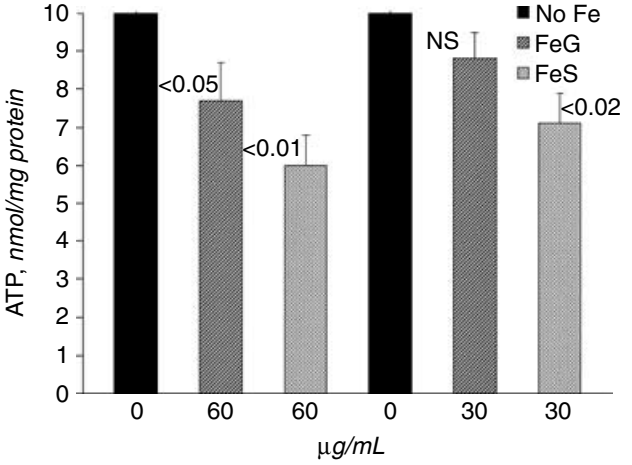

Fig. 2. Proximal tubular segment adenosine triphosphate (ATP) concentrations with low dose (30 and 60 μg/mL) iron sucrose (FeS) and iron gluconate (FeG) exposures. Both drugs caused significant ATP depressions at the 60 μg/mL concentration. Each drug also tended to depress ATP concentrations even at the 30 μg/mL dosage, but only the FeS result achieved statistical significance ($P < 0.02$ vs. controls).

Calculations and statistics

All values are presented as means ± 1 SEM. Statistical comparisons were made by paired or unpaired Student *t* testing, as per the nature of the experiment. If multiple comparisons were made, the Bonferroni correction was applied.

RESULTS

Isolated tubule experiments

Proximal tubule ATP concentrations in response to 125 to 1000 μg/mL iron exposures. As shown in Figure 1, each of the test iron preparations caused dose dependent reductions in tubule ATP concentrations. The FeOS and FeD curves did not significantly differ, and statistically significant ATP reductions were observed at only the highest tested concentration (1000 μg/mL of iron; $P < 0.04$ vs. their respective controls). In contrast, FeS and FeG each induced steep dose-response curves, clearly much more severe than those observed with either FeOS or FeD. FeS caused the most severe ATP depressions, with ~50% greater ATP losses being observed vs. their corresponding FeG results ($P < 0.0001$ in an overall comparison between paired concentrations).

% LDH release with the 125 to 1000 μg/mL iron dosage range. The 95% confidence band for % LDH release for control tubules was 8% to 13%. In the above-described 30-minute titration experiments, only FeS raised % LDH release above this normal range, but this was observed only at the two highest test concentrations (41 ± 3%, 14 ± 1%, and 13 ± 1% with 1000 μg/mL, 500 μg/mL, and 250 μg/mL iron doses, respectively) (data not shown). Each of the other test compounds (FeD, FeG, and FeOS) failed to raise % LDH release above control values (≤13%) even with application of 1000 μg/mL iron concentrations.

pH controls for high dose Venofer additions. Addition of alkaline sucrose solution (pH 10.5), did not reproduce FeS cytotoxic effects. First, it tended to *raise*, rather than lower, tubule ATP concentrations (pH 10.5, 8.4 ± 0.6; controls, 8.0 ± 0.5 nmol/mg protein). Second, % LDH release was 14 ± 1% with alkaline sucrose incubation, compared to 41 ± 3% with the 1000 μg/mL FeS addition. Third, even the highest test dose of FeS (1000 μg/mL) had only a small effect on tubule suspension pH, raising it from 7.44 to 7.8. Lesser amounts of FeS addition had no discernible pH effect.

ATP concentrations and LDH release with "low dose" (30 and 60 μg/mL) iron concentrations. As shown in Figure 2, even when added in a 30 or 60 μg iron/mL dose, FeS still caused statistically significant reductions in tubule ATP concentrations, compared to co-incubated control tubules. FeG also lowered ATP at these two iron concentrations, but the result was statistically significant only at the 60 μg/mL concentration (Fig. 2). None of these incubations caused a significant increase in LDH release (range for controls and iron compounds, 9% to 11%).

In vivo experiments

MDA levels following parenteral iron treatment. As shown in Figure 3A, each of the iron compounds induced statistically significant plasma MDA increments, rising well above the upper 95% confidence limit (shown by horizontal line) for normal plasma MDA values. The plasma MDA increase was ~2× as great with FeD, compared

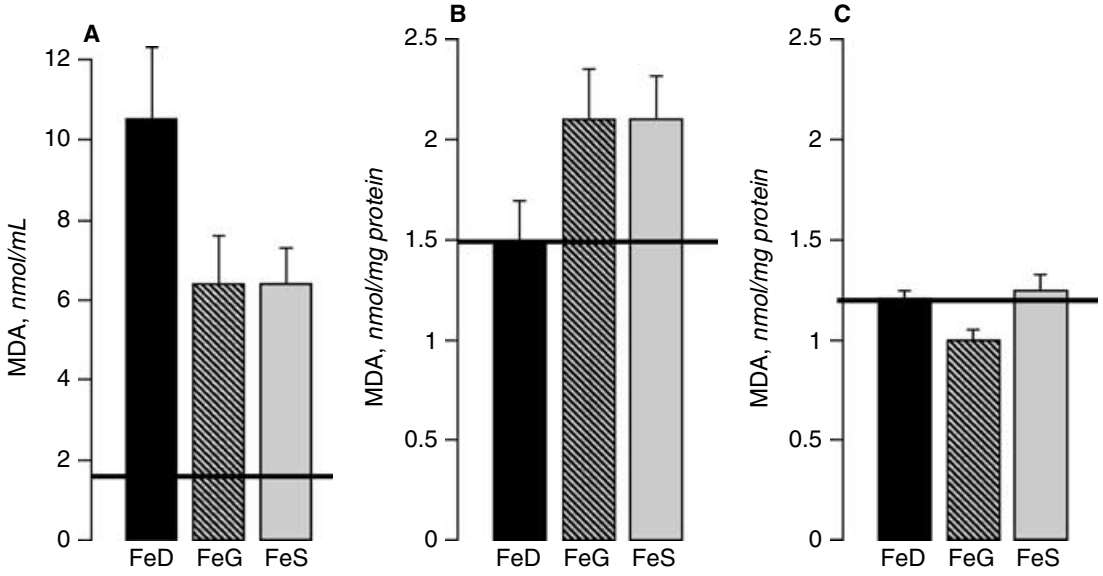

Fig. 3. Malondialdehyde (MDA) concentrations in plasma (*A*), renal cortex (*B*), and apical myocardium (*C*) following intravenous iron dextran (FeD), iron gluconate (FeG), or iron sucrose (FeS) injection. Whereas FeD caused the greatest plasma MDA increase, it failed to induce renal cortical lipid peroxidation (horizontal lines are the upper limit of the 95% confidence interval for normal plasma or tissue MDA levels). Minimal cardiac lipid peroxidation resulted, and it was significant only with FeS treatment. Thus, these results indicate that while intravenous irons can cause in vivo lipid peroxidation, the degrees to which they do so are both compound, and target tissue, dependent.

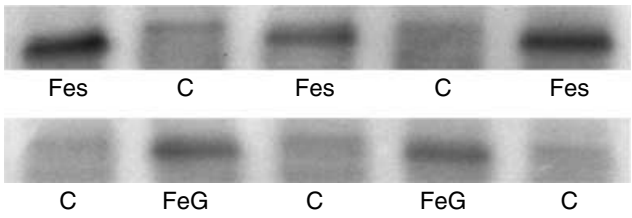

Fig. 4. Western blotting of renal cortex for tissue ferritin after the 1 week, every other day, iron treatment protocols. Only minimal ferritin expression was seen in control kidney samples (C). Iron sucrose (FeS) treatment clearly increased tissue ferritin expression. A lesser, but still significant, increase in ferritin was apparent following iron gluconate (FeG) treatment. In contrast, neither iron dextran (FeD) nor iron oligosaccharide (FeOS) caused any ferritin increments (not depicted; see Fig. 5).

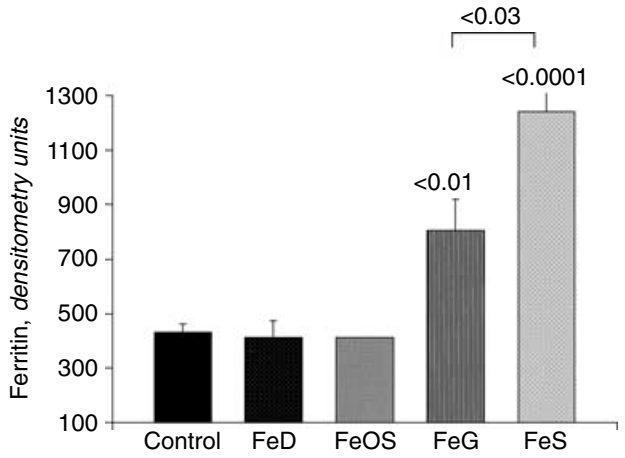

Fig. 5. Western blot densitometric analysis of renal cortical ferritin expression. Neither iron dextran (FeD) nor iron oligosaccharide (FeOS) induced any change in renal cortical ferritin levels, as assessed by Western blotting. In contrast, iron gluconate (FeG) and iron sucrose (FeS) each raised renal cortical ferritin levels, compared to the control tissue samples ($P < 0.01$ and $P < 0.0001$, respectively). The increase was significantly greater with FeS vs. FeG ($P < 0.03$).

to either FeG or FeS treatment. However, in striking contrast to plasma (where FeD caused the greatest MDA increases), in renal cortex, MDA increments resulted from only FeG and FeS, but not FeD injection (Fig. 3B). The heart was relatively resistant to iron-mediated lipid peroxidation, as only FeS caused any increase in cardiac MDA values (above the 95% confidence limits) (Fig. 3C). Thus, in composite, these MDA results indicate that while all of the test iron compounds can evince lipid peroxidation, the degree to which this occurs depends on the particular tissue target (e.g., plasma, kidney, or heart) and the particular drug (FeS, FeG, or FeD) involved.

Renal cortical ferritin and HO-1 protein expression. Slight ferritin expression was seen in control renal cortical tissue samples (Fig. 4). FeG and FeS each increased ferritin levels, rising ~2× and ~4× over control values, respectively (Fig. 5). The increase was statistically greater with FeS, in comparison to FeG treatment ($P < 0.03$). Neither FeD nor FeOS caused any discernible ferritin increase (i.e., above control values).

In contrast to ferritin, none of the treatments caused any clearly discernible change in HO-1 protein expression (data not shown). In contrast, renal cortex obtained 18 hours postinduction of rhabdomyolysis-induced acute

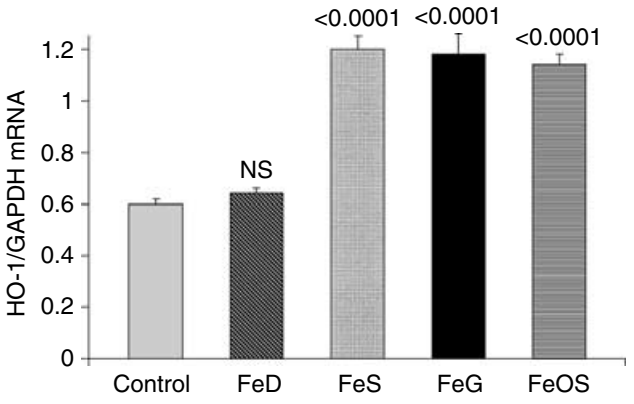

Fig. 6. Heme oxygenase- 1(HO-1) mRNA levels in renal cortex 4 hours following intravenous iron dextrose (FeD), iron sucrose (FeS), iron gluconate (FeG), or iron oligosaccharide (FeOS) injection. Excepting FeD, all of the iron preparations induced an approximate doubling of HO-1 mRNA levels, consistent with the induction of oxidative stress in renal cortex.

renal failure showed prominent HO-1 induction (seen at 32 kD), confirming the adequacy of the employed Western blot analysis.

Renal cortical HO-1 mRNA expression. As shown in Figure 6, by 4 hours postinjection, FeS, FeG, and FeOS each caused an approximate doubling of HO-1 mRNA levels ($P < 0.0001$ for each vs. controls). In striking contrast, FeD caused no change in renal cortical HO-1 mRNA, with values being virtually identical to control values. Of note, these results paralleled the above-described MDA values, whereas both FeG and FeS caused an approximate 30% increase in renal cortical MDA, FeD did not raise renal MDA levels.

Renal histology. There was no definitive evidence of renal histologic injury (Figs. 7 to 9), as discerned by light microscopy (which was the reason for undertaking the electron microscopy analyses). FeS, and to a lesser extent FeG (but not FeD or FeOS) did induce histologic damage, as assessed by electron microscopy. The most notable change was glomerular iron accumulation, taking the form of electron dense aggregates which were most prominent in the mesangium and in endothelial cells. Additionally, occasional large iron deposits could be found in podocyte cell bodies (Fig. 8B). The foot processes remained well preserved. Glomerular endothelial deposits were associated with moderate to marked endothelial cell swelling, observed in ~40% of glomerular capillary loops (for example, see Fig. 7B). This led to focal or diffuse obliteration of endothelial fenestrations along the glomerular basement membrane. Noteworthy was the complete absence of any discernible iron accumulation (and attendant endothelial injury) with either FeD or FeOS treatment (e.g., Fig. 9B). In none of the samples analyzed by electron microscopy could proximal tubular iron accumulation, or histologic evidence of tubular injury, be observed. However, with FeS treatment, occasional empty vacuoles were observed in proximal tubular cells (suggesting prior iron trafficking through proximal tubular cells).

HK-2 cell experiments

% LDH release. After 3-day exposures to 100 μg/mL of FeS, a marked increase in % LDH release was apparent (Fig. 10B). FeG caused only a slight, but significant, increase in LDH release ($P < 0.05$). Conversely, neither FeD nor FeOS increased LDH release above control values.

Cytochrome c release. As a second marker of cellular/mitochondrial injury, cytochrome c levels remaining in adherent HK-2 cells in the above experiment were assessed. As shown (Fig. 10A), there was no discernible loss of cellular cytochrome c following either FeG, FeD, or FeOS treatment. However, cytochrome c was decreased by ~95% in the cell pellets obtained following FeS treatment.

Gross appearance of HK-2 cells following incubation with test iron preparations. The cells which were harvested for cytochrome c analysis appeared grossly different in visual appearance. There was a distinct, dark brownish discoloration of cells treated with FeG and FeS, with the latter inducing the most profound color change (Fig. 11). In contrast, neither FeOS nor FeD caused any cell color change. Thus, gross cell appearance suggested the following gradations of cell iron accumulation: FeS > FeG vs. none with FeOS or FeD (paralleling degrees of toxicity).

Electron microscopic evaluation of HK-2 cells. The normal appearance of control HK-2 cells is shown in Fig. 12A. In particular, there were frequent plasma membrane microvilli seen, and in some instances, tight junctions were observed (data not shown). Cells incubated with FeS manifested marked accumulation of electron-dense deposits within the cytoplasm, indicative of iron uptake (Fig. 12B). These deposits appeared to be in membrane-associated vacuoles. FeG treatment also led to iron uptake (Fig. 13A), albeit to a lesser degree than observed with FeS. The iron deposits with FeS appeared larger than those seen with FeG, and in some cases, the former took on a "tangled rope like" appearance within cytosolic vacuolar structures (e.g., Fig. 14A). In contrast, neither FeD (Fig. 13B) nor FeOS (Fig. 14B) demonstrated *any* cytoplasmic iron accumulation. Despite the evidence that mitochondria were targets of injury (based on cytochrome c release and in vivo mitochondrial degeneration), the iron treatments did not seem to perturb HK-2 mitochondrial structure (suggesting rapid mitochondrial disruption/elimination following 3 days of injury).

Assessment of whether parenteral iron loading induces tubular cytoresistance. As shown in Figure 15, tubules

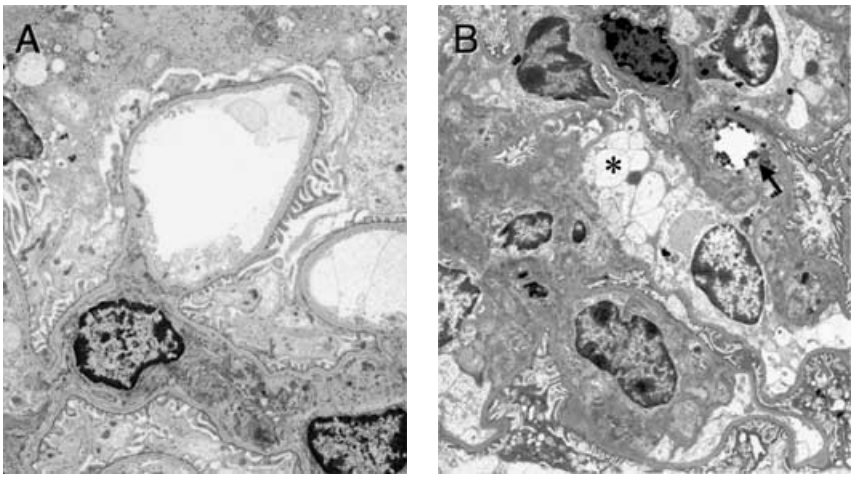

Fig. 7. Electron micrograph of normal mouse renal cortex (*A*) and renal cortex after 1 week of every other day iron sucrose (FeS) treatment (*B*). Note the presence of normal endothelial fenestrations in the control tissue (A). FeS treatment caused variable degrees of endothelial cell swelling (*), resulting in focal narrowing or closure of capillary loops with loss of fenestrae. Foci of iron deposition were also observed in endothelial cells (e.g., see arrow).

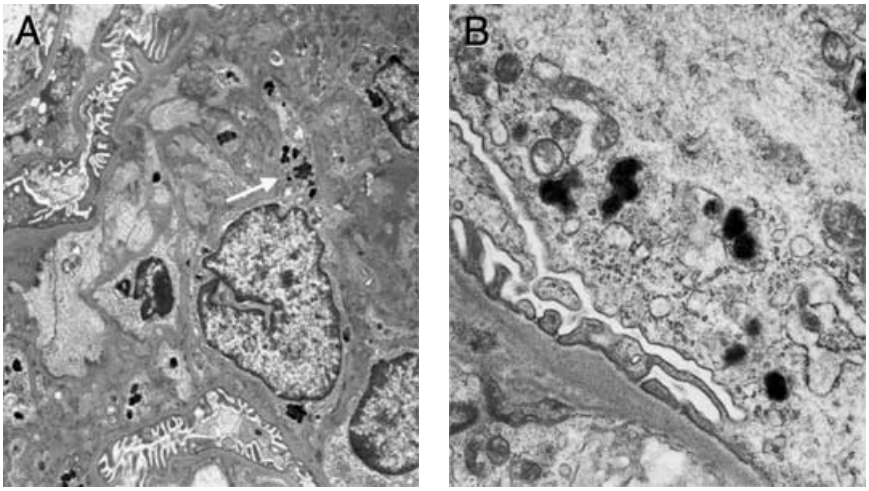

Fig. 8. Electron micrographs of mouse renal cortex following 1 week of every other day iron sucrose (FeS) treatment. Extensive black deposits of iron are present in the mesangium, and to a lesser extent in endothelial cells (*A*). Focal iron deposition was also observed in podocytes but with preservation of foot processes (*B*).

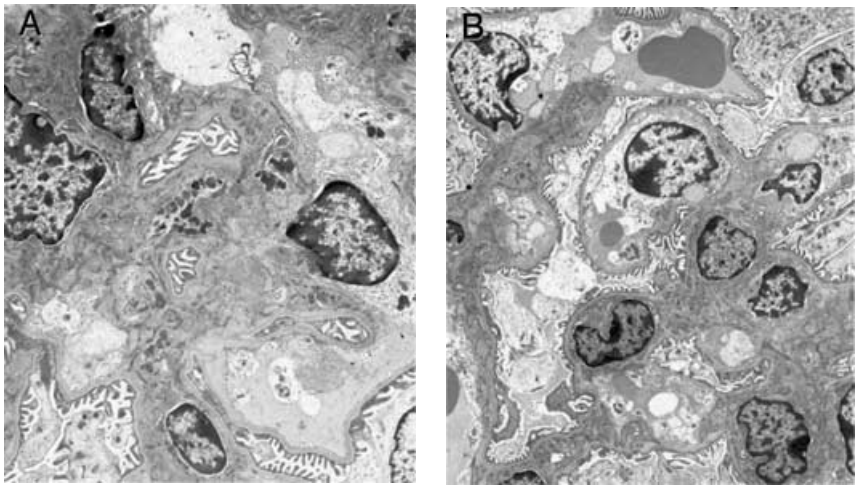

Fig. 9. Electron micrograph of mouse renal cortex following 1 week of every other day iron gluconate (FeG) (*A*) or iron dextran (FeD) (*B*) treatment. FeG treatment led to extensive iron deposition in glomerular structures (A). Conversely, FeD injections resulted in no discernible iron deposition. Not depicted, iron oligosaccharide (FeOS) injections also were not associated with discernible iron accumulation in kidney, as assessed by electron microscopy.

isolated from FeS and control mice had identical degrees of viability under control incubation conditions (~10% LDH release). No significant differences in tubule injury were apparent with either the antimycin A or with the hypoxic challenge. In contrast, the FeHQ challenge caused significantly less tubule injury in FeS pretreated mice, with an approximate 50% decrease in FeHQ-mediated LDH release being observed (i.e., % LDH released above control values 15% vs. 7% for control vs. FeS pretreated tubules, respectively). Thus, these results indicated that

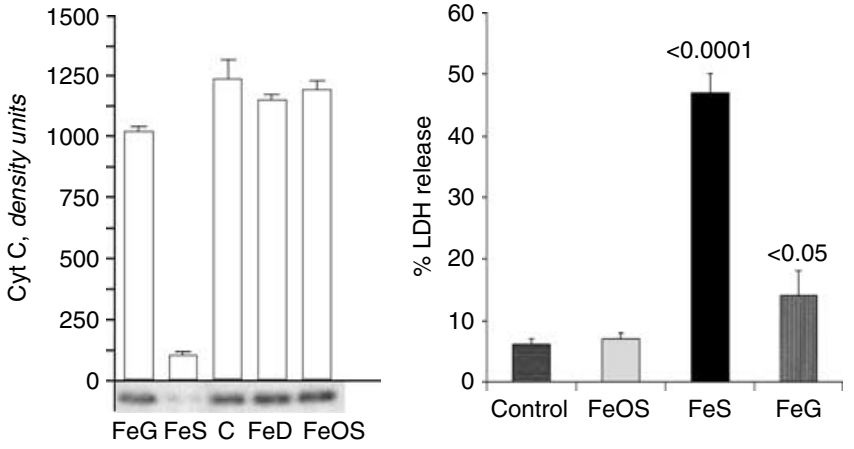

Fig. 10. Cytochrome c (cyt c) (*A*) and lactate dehydrogenase (LDH) (*B*) loss from human kidney (HK-2) cells in response to 3-day 100 µg/mL iron exposures. Cytochrome c levels remained at, or near, control values with iron dextran (FeD), iron oligosaccharide (FeOS), or iron gluconate (FeG) treatment. Conversely, iron sucrose (FeS) treatment caused massive loss of cellular cytochrome c. As shown in (B), FeS, and to a lesser extent FeG, caused significant LDH release, compared to control values.

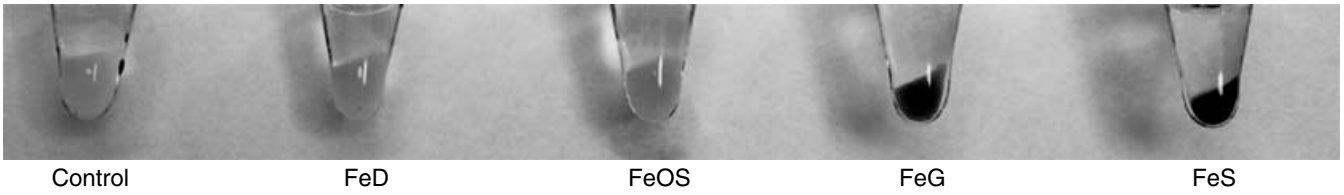

Fig. 11. Pelleted human kidney (HK-2) cells after 3 days of control incubations, or exposure to 100 µg/mL of either iron dextran (FeD), iron oligosaccharide (FeOS), iron gluconate (FeG), or iron sucrose (FeS) treatment. The gross appearances of the FeD- and FeOS-exposed cells were identical to that of the control cells. However, FeS, and to a lesser extent FeG, caused prominent iron staining. Subsequent electron microscopy analysis confirmed intracellular FeS and FeG iron accumulation.

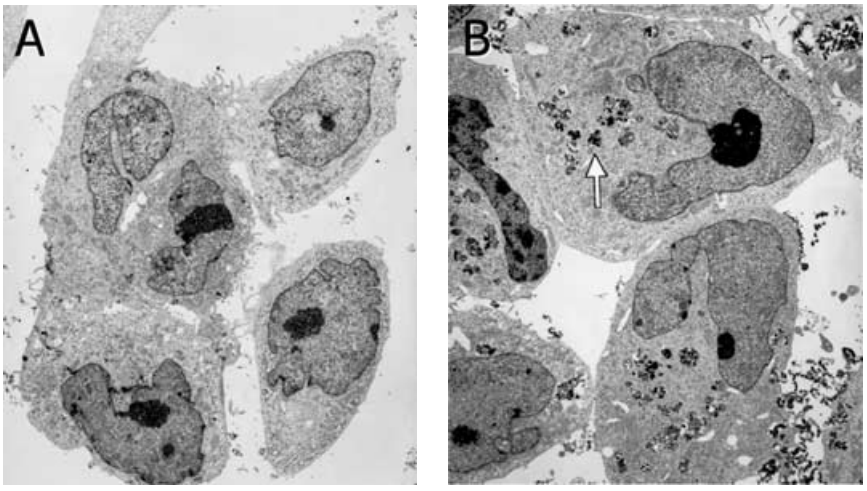

Fig. 12. Electron micrographs of human kidney (HK-2) cells after control incubation (*A*) or following 3-day iron sucrose (FeS) treatment (*B*). As shown (B, see arrow for example), there was extensive iron uptake by the HK-2 cells. In the bottom right hand corner of (B), iron appears to be in the internalization process.

FeS iron was capable of impacting in vivo proximal tubular cells.

DISCUSSION

Although several reports have raised concerns of potential adverse consequences of parenteral iron administration to renal disease patients [6–12], it remains unclear as to whether differing degrees of cytotoxicity exist amongst currently employed iron preparations. Such a possibility is suggested by the fact that variable carbohydrate structures are used to "shield" each compound's iron content; thus, formulation differences might impact iron's cytotoxic effects. Iron-induced oxidative stress is widely accepted as a critical mediator of diverse forms of glomerular and tubulointerstitial renal disease [26–29]. Thus, intravenous iron administration could potentially contribute to renal disease progression. Oxidative stress can also exacerbate systemic inflammation [18–22] and atherogenesis [12–17]. That these latter two conditions negatively impact patient survival on dialysis [13, 18, 23] underscores parenteral iron's potential to exert

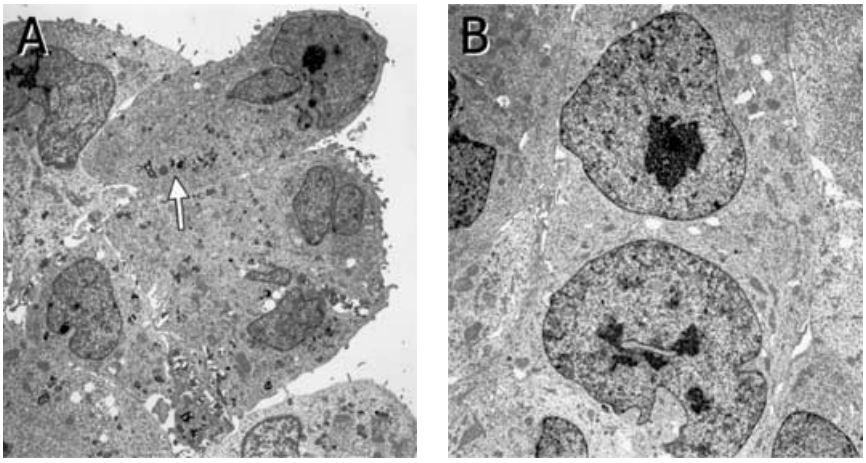

Fig. 13. Electron micrographs of human kidney (HK-2) cells after incubation for 3 days with either iron gluconate (FeG) (*A*) or iron dextran (FeD) (*B*). Cells treated with FeG had obvious iron incorporation (see arrow for an example). Conversely, no iron uptake was seen following FeD treatment.

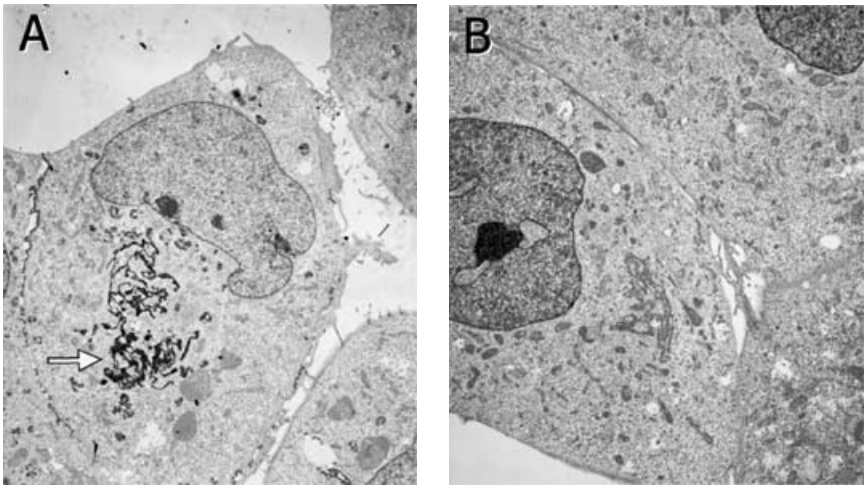

Fig. 14. Electron micrographs of human kidney (HK-2) cells after 3 days of either iron sucrose (FeS) (*A*) or iron oligosaccharide (FeOS) treatments (*B*). In addition to the punctate intracellular FeS iron depicted in Fig. 12B, in some cells the iron assumed a tangled filamentous appearance (A). As shown in the right hand panel, FeOS exposure [as with iron dextran (FeD), Fig. 13B] caused no discernible intracellular iron uptake.

adverse effects. In light of these considerations, this study addressed whether relative differences in toxicity exist among four representative parenteral iron formulations, and if so, what are some potential underlying determinants thereof.

In a previous study from this laboratory [5], we posited that marked differences in cytotoxicity do, in fact, exist among four intravenous iron formulations, with the following relative rank order of toxicity: FeS $\gg$ FeG $\gg$ FeD = FeOS. However, this conclusion was based solely on in vitro experiments which predominantly relied on cellular LDH release as a biologic readout. Furthermore, those experiments were, in large part, conducted using supratherapeutic iron concentrations. Thus, at least two important questions remained to be addressed: (*1*) might differential iron toxicity also be expressed at clinically relevant iron concentrations? and (*2*) might toxicologic differences, as defined in vitro, also be expressed in an in vivo setting?

We previously noted that FeS toxicity had as a correlate acute mitochondrial dysfunction [5]. This conclusion was based on findings that cell exposure to 1 mg/mL of FeS iron caused rapid and profound (~90%) ATP depletion in freshly isolated mouse proximal tubules. NaK-ATPase inhibition (ouabain) did not abrogate FeS-induced ATP declines, indicating that decreased mitochondrial ATP production, rather than increased ATP consumption, was likely responsible. The present study expands on these initial observations in two important ways: (*1*) relative degrees of mitochondrial dysfunction/ATP declines have been delineated for each of the above iron formulations; and (*2*) a full dose-response relationship for each test agent has been defined. Of note, the three lowest test iron dosages (30, 60, and 125 μg iron/mL) fall within a clinically achievable plasma iron concentration range (e.g., ~150 μg/mL following ~500 mg intravenous iron infusion). As shown in Figure 1, the extent of ATP reductions which followed iron exposures indicated an identical rank order of compound toxicity to that which we previously reported (i.e., FeS $\gg$ FeG $\gg$ FeD = FeOS) [5]. Furthermore, FeS and FeG caused statistically significant ATP declines even with a 30 to 60 μg/mL iron

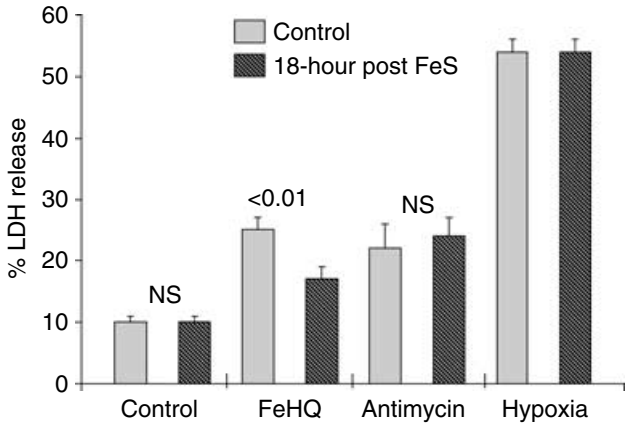

Fig. 15. Injury to proximal tubular segments harvested from either control mice or mice treated 18 hours previously with intravenous iron sucrose (FeS). There was no difference in cell viability between control tubules and prior FeS-exposed tubules under normal incubation conditions. FeS pretreatment induced significant protection against in vitro iron [iron-hydroxyquinoline (HQ)]-induced oxidant injury ($P < 0.01$). However, this protection appeared to be specific for oxidant injury, as no difference in lactate dehydrogenase (LDH) release was apparent between the groups which were subjected to the antimycin A or hypoxic challenges.

concentration range. Conversely, FeD and FeOS required 1 mg/mL of iron to induce even minimal ATP declines. Thus, these findings indicate that up to 30-fold differences in relative toxicity exist amongst selected intravenous iron preparations (e.g., FeS vs. FeD).

In a "Letter to the Editor" [46], Van Wyck strongly opined, but in the absence of supporting data, that the alkaline pH (~10.5) of the stock FeS solution was the explanation for our previous findings [5] of preferential FeS cytotoxicity. Indeed, we, as well as others, have previously reported that alkaline conditions can decrease cell viability, particularly when those cells are challenged with superimposed attack (e.g., hypoxia) [46]. However, the current results conclusively rule out Van Wyck's "pH hypothesis." First, even at the highest dosage employed (1 mg/mL), FeS addition to isolated tubule suspensions induced a relatively small change in tubule suspension pH (rising from 7.44 to 7.80). Second, with subsequent serial dilutions of the FeS stock solution (from 500 → 30 μg/mL), no discernible changes in isolated tubule suspension pH were detected, and yet dramatic ATP reductions still occurred (dissociating these two events). Finally, alkaline sucrose addition to isolated tubules (to recreate FeS *maximal* pH effect) tended to raise, rather than lower, tubule ATP content and it failed to reproduce FeS cytotoxic effect (i.e., LDH release). Clearly then, as discussed below, alternative mechanisms for preferential FeS cytotoxicity must exist.

To gain further support for the concept that parenteral iron toxicity is expressed at, or potentially mediated by, mitochondrial injury, a second end point of mitochondrial damage, notably cytochrome c release, was assessed in cultured proximal tubule (HK-2) cells following a 3-day 100 μg/mL iron challenge. Recent work from this laboratory [31] has demonstrated that following iron-induced mitochondrial toxicity, cytochrome c rapidly leaves the mitochondrion, traverses the cytosol, crosses an intact plasma membrane, and eventuates in the extracellular space [31]. Thus, total cellular (and not just mitochondrial) cytochrome c loss serves as an index of mitochondrial damage. As shown in Figure 10, FeS treatment induced almost complete cytochrome c loss from HK-2 cells. In contrast, FeG caused a nonsignificant trend toward cell cytochrome c reductions, whereas FeD and FeOS had no discernible effect. These results were paralleled by the accompanying LDH release data: 45%, 18%, and 5% for FeS, FeG, and FeD/FeOS, respectively. Clearly then, these HK-2 cell results fully support the relative toxicity profiles gleaned from the above isolated mouse proximal tubule experiments (FeS > FeG > FeD or FeOS), and further indicate that the mitochondrion is a critical intracellular iron target. That HK-2 cytotoxicity could again be expressed at a clinically achievable iron concentration underscores the potential clinical relevance of these observations.

The cells which were collected from the above described HK-2 cell experiments provided a striking, and unexpected, clue as to why the four test iron agents exert differential cytotoxicity. As depicted in Figure 11, varying amounts of brownish cell staining was apparent, with its degree exactly paralleling the rank order toxicity data: FeS ≫ FeG ≫ FeD = FeOS = controls. To determine whether these color changes simply reflected iron adherence to, or staining of, the plasma membrane vs. cellular iron incorporation, electron microscopic analyses were performed. As illustrated in Fig. 12B, FeS iron was clearly internalized within HK-2 cells. FeG induced similar, albeit less pronounced, intracellular iron loading (Fig. 13A). In contrast, there was a complete lack of iron internalization following 3 days of either FeD or FeOS treatment. Thus, these findings strongly suggest that the differential cytotoxicity of these test agents reflects their differing capacities to gain intracellular access. The reason for this differential uptake remains unknown. In pilot data not presented, FeS uptake appeared to be independent of a possible sucrose transport pathway, given that excess sucrose addition to FeS-challenged HK-2 cells neither diminished FeS uptake or the resultant LDH release.

The final goal of this study was to determine whether in vivo correlates of parenteral iron toxicity might exist. To achieve this goal, a relatively large amount of iron (2 mg) was employed. While this obviously exceeds a clinically relevant dosage, it should be noted that acute, rather than chronic, toxicity was sought. Furthermore, a large dose was employed to facilitate potential iron detection/localization in renal histologic sections. Based on

these experiments, it is clear that in vivo iron toxicity can, indeed, be expressed, as delineated by the following observations. First, lipid peroxidation results, as indicated by marked plasma MDA increments. Second, these plasma MDA increases can have tissue correlates, as denoted by renal cortical, and to a much lesser extent myocardial, lipid peroxidation. Third, patterns and degrees of lipid peroxidation differ, according to the iron compound employed and the specific target organ involved (see **Results** section). Fourth, renal cortical oxidative stress was confirmed by documenting HO-1 mRNA increases with different test iron compounds. Surprisingly, no corresponding HO-1 protein increments resulted, suggesting that despite the mRNA increases, a failure of normal HO-1 message translation may result. Finally, renal cortical iron exposure was confirmed by documenting increased tissue ferritin content following FeS and FeG (but not FeD or FeOS) treatment. It is noteworthy that, as with the in vitro results, differential in vivo iron-mediated "toxicity" was apparent. For example, the rank order of renal cortical ferritin induction paralleled the in vitro toxicity profiles (FeS > FeG > FeD or FeOS). Furthermore, FeS and FeG each raised renal cortical MDA and HO-1 mRNA, whereas FeD failed to induce either of these two results. Clearly, then, differential, and seemingly parallel, toxicities can be expressed in both in vivo and in vitro settings.

If differential parenteral iron toxicity is, indeed, dependent on differing degrees of iron uptake (as suggested by the HK-2 cell experiments), it would be predicted that greater renal cortical iron accumulation would follow FeS and FeG, vs. FeD or FeOS treatments. Indeed, the electron microscopy evaluations of renal cortex obtained following parenteral iron therapy were entirely consistent with this hypothesis. FeS, and to a somewhat lesser extent FeG, treatment led to prominent iron accumulation within the mesangium, glomerular endothelial cells, and to a lesser extent, in podocytes. Conversely, FeD or FeOS injections caused no discernible renal iron deposits. That glomerular endothelial swelling/fenestrae loss was a correlate of FeS, and to a lesser extent of FeG, accumulation indicates the pathogenic potential of this process. Somewhat surprising was the lack of proximal tubular iron deposition, at least as could be discerned by electron microscopy. However, there can be little doubt that intravenous iron injections can impact proximal tubular cell homeostasis, given that tubules isolated from FeS-treated mice manifested partial cytoresistance to subsequent FeHQ-induced oxidant attack. Noteworthy in this regard are recent findings of Nath et al [26]. These workers demonstrated that recurrent iron-induced oxidant injury, although eliciting transient cytoresistance, ultimately culminates in chronic tubulointerstitial nephritis [26]. These observations, coupled with the findings that FeS induced glomerular iron deposits, endothelial injury, and tubular cytoresistance, raise the concern that chronic parenteral iron treatment could potentially evoke renal damage.

CONCLUSION

The present study provides additional support for the concept that parenteral iron formulations have intrinsic cytotoxic potentials, and that the degrees to which these toxicities are expressed is critically dependent on the nature of carbohydrate polymers employed. The latter appear to determine cellular iron uptake, both in vivo and in vitro, and hence the capacity of these compounds to induce their cytotoxic effects. That these compound(s) can up-regulate renal cortical HO-1 mRNA and tissue ferritin, and induce tissue lipid peroxidation, indicate that oxidative injury (e.g., directed at mitochondria) is the likely pathogenic pathway by which parenteral iron toxicity results. While the clinical relevance of these findings remains speculative at this time, caution would appear to be warranted when administering these compounds to patients with renal disease. Indeed, given that intravenous iron can damage normal kidneys, its potential nephrotoxicity might be even greater in the presence of active nephropathy. Of considerable interest in this regard, Agarwal et al [48] recently reported that FeS administration to chronic renal disease patients increases both proteinuria and proximal tubular enzymuria. This serves to underscore the above concerns.

ACKNOWLEDGMENTS

This work was supported by research grants from the National Institutes of Health (DK38432 and DK-54200). The authors thank Charles E. Alpers, M.D., University of Washington, Seattle, Washington, for his assistance with interpreting renal electron microscopy. We also thank the staff of the Electron Microscopy Department of the Fred Hutchinson Cancer Research Center for their expertise and support.

Reprint requests to Richard A. Zager, M.D., Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. N; Room D2-190, Seattle, WA 98109. E-mail: dzager@fhcrc.org

REFERENCES

1. Nissenson AR, Strobos J: Iron deficiency in patients with renal failure. *Kidney Int* 59(Suppl 69):S18–S21, 1999
2. National Kidney Foundation: DOQI Clinical Practice Guidelines for the Treatment of Anemia of Chronic Renal Failure. *Am J Kid Dis* 37(Suppl 1):S182–S238, 2001
3. MacDougall IC, Horl WH, Jacobs C, *et al*: European Best Practice Guidelines 6–8: Assessing and optimizing iron stores. *Nephrol Dial Transplant* 15(Suppl 4):20–32, 2000
4. Faich G, Strobos J: Sodium ferric gluconate complex in sucrose: Safer intravenous iron therapy than iron dextrans. *Am J Kid Dis* 37:743–749, 1999
5. Zager RA, Johnson ACM, Hanson SY, Wasse H: Parenteral iron formulations: A comparative toxicologic analysis and mechanisms of cell injury. *Am J Kidney Dis* 40:90–103, 2002
6. Salahudeen AK, Oliver B, Bower JD, Oberts LJ: Increase in plasma esterified F-2 isoprostanes following intravenous iron infusion in patients on hemodialysis. *Kidney Int* 60:1525–1531, 2001

7. Roob JM, Khoschsorur G, Tiran A, *et al*: Vitamin E attenuates oxidative stress induced by intravenous iron in patients on hemodialysis. *J Am Soc Nephrol* 8:475–486, 1997
8. Lim PS, Wei YH, Yu YL, Kho B: Enhanced oxidative stress in haemodialysis patients receiving intravenous iron therapy. *Nephrol Dialysis Transplant* 14:2680–2687, 1999
9. Cavdar C, Temiz A, Yenicerioglu Y, *et al*: The effects of intravenous iron treatment on oxidant stress and erythrocyte deformability in hemodialysis patients. *Scand J Urol Nephrol* 37:77–82, 2000
10. Rooyakkers TM, Stroes ES, Kooistra MP, *et al*: Ferric saccharate induces oxygen radical stress and endothelial dysfunction in vivo. *Eur J Clin Invest* 32(Suppl 1):9–16, 2002
11. Sengoelge G, Kletzmayr J, Ferrara I, *et al*: Impairment of trans-endothelial leukocyte migration by iron complexes. *J Am Soc Nephrol* 14:2639–2644, 2002
12. Drueke T, Witko-Sarsat V, Massy Z, *et al*: Iron therapy, advanced oxidation protein products, and carotid artery intima-media thickness in end-stage renal disease. *Circulation* 106:2212–2217, 2002
13. Becker BN, Himmelfarb J, Henrich WL, Hakim RM: Re-assessing the cardiac risk profile in chronic hemodialysis patients: A hypothesis on the role of oxidant stress and other non traditional cardiac risk factors. *J Am Soc Nephrol* 8:475–486, 1997
14. Gackowski D, Kruszewski M, Jawien A, *et al*: Further evidence that oxidative stress may be a risk factor responsible for the development of atherosclerosis. *Free Radical Biol Med* 31:542–547, 2001
15. Ponraj D, Makjanic J, Thong PS, *et al*: The onset of atherosclerotic lesion formation in hypercholesterolemic rabbits is delayed by iron depletion. *FEBS Lett* 459:218–222, 1999
16. Daugherty A: Atherosclerosis: Cell biology and lipoproteins. *Curr Opin Lipid* 8:11–12, 1997
17. Corti MC, Gaziano M, Hennekens CH: Iron status and risk of cardiovascular disease. *Ann Epidemiol* 7:62–68, 1997
18. Spittle MA, Hoenich NA, Handelman GJ, *et al*: Oxidative stress and inflammation in hemodialysis patients. *Am J Kidney Dis* 38:1408–1413, 2001
19. Rahman I, MacNee W: Regulation of redox glutathione levels and gene transcription in lung inflammation: therapeutic approaches. *Free Rad Biol Med* 28:1405–1420, 2000
20. Haddad JJ: Antioxidant and prooxidant mechanisms in the regulation of redox(y)-sensitive transcription factors. *Cellular Signalling* 14:879–897, 2002
21. Xiong S, She H, Takeuchi H, *et al*: Signaling role of intracellular iron in NF-kappa B activation. *J Biol Chem* 278:17646–17654, 2003
22. Sukamoto H: Iron regulation of hepatic macrophage TNF alpha expression. *Free Radical Biol Med* 32:309–313, 2002
23. Owen WF, Lowrie EG: C reactive protein as an outcome predictor for maintenance hemodialysis patients. *Kidney Int* 54:627–636, 1998
24. Zoccali C, Benedetto FA, Mallamaci F, *et al*: Inflammation is associated with carotid atherosclerosis in hemodialysis patients. CREED Investigators. Cardiovascular Risk Extended Evaluation in Dialysis Patients. *J Hypertens* 18:1207–1213, 2000
25. Iseki K, Tozawa M, Yoshi S, Fukiyama K: Serum C-reactive protein (CRP) and risk of death in chronic dialysis patients. *Nephrol Dial Transplant* 14:1956–1960, 1999
26. Nath KA, Vercellotti GM, Grande JP, *et al*: Heme protein-induced chronic renal inflammation: Suppressive effect of induced heme oxygenase-1. *Kidney Int* 59:106–117, 2001
27. Fernandez-Real JM, Lopez-Bermejo A, Ricart W: Cross-talk between iron metabolism and diabetes. *Diabetes* 51:2348–2354, 2002
28. Shah SV: The role of reactive oxygen metabolites in glomerular disease. *Ann Rev Physiol* 57:245–262, 1995
29. Baliga R, Ueda N, Walker PD, Shah SV: Oxidant mechanisms in toxic acute renal failure. *Am J Kidney Dis* 29:465–477, 1997
30. Zager RA, Burkhart KM, Conrad DS, Gmur DJ: Iron, heme oxygenase, and glutathione effects on myohemoglobinuric proximal tubular injury. *Kidney Int* 48:1624–1634, 1995
31. Zager RA, Johnson ACM, Hanson SY: Proximal tubular cytochrome c efflux: Determinants and a potential marker of mitochondrial injury. *Kidney Int* (in press)
32. Zager RA: Hyperthermia: Effects on renal ischemic/reperfusion injury in the rat. *Lab Invest* 63:360–369, 1990
33. Zager RA, Schimpf BA, Gmur DJ: Physiologic pH: Effect on posthypoxic proximal tubular injury. *Circ Res* 72:837–846, 1993
34. Gamelin LM, Zager RA: Evidence against oxidant injury as a critical mediator of post ischemic acute renal failure. *Am J Physiol* 255:F450–F460, 1988
35. Johnson AC, Yabu JM, Hanson S, *et al*: Experimental glomerulopathy alters renal cortical cholesterol, SR-B1, ABCA1, and HMG CoA reductase expression. *Am J Pathol* 162:283–291, 2003
36. Nath KA, Balla G, Vercellotti GM, *et al*: Induction of heme oxygenase is a rapid, protective response in rhabdomyolysis in the rat. *J Clin Invest* 90:267–270, 1992
37. Carraway MS, Ghio AJ, Taylor JL, Piantadose CA: Induction of ferritin and heme oxygenase-1 by endotoxin in the lung. *Am J Physiol* 275:L583–L592, 1998
38. Zager RA, Shah VO, Shah HV, *et al*: The mevalonate pathway during acute tubular injury: Selected determinants and consequences. *Am J Pathol* 161:681–692, 2002
39. Chen Z, Friedrich GA, Soriano P: Transcriptional enhancer factor 1 disruption by a retroviral gene trap leads to heart defects and embryonic lethality in mice. *Genes Develop* 8:2293–2301, 1994
40. Ryan MJ, Johnson G, Kirk J, *et al*: HK-2: An immortalized proximal tubule epithelial cell line from normal adult human kidney. *Kidney Int* 45:48–57, 1994
41. Mancini M, Anderson BO, Caldwell E, *et al*: Mitochondrial proliferation and paradoxical membrane depolarization during terminal differentiation and apoptosis in a human colon carcinoma cell line. *J Cell Biol* 138: 449–469, 1997
42. Zager RA: Heme protein-induced tubular cytoresistance: Expression at the plasma membrane level. *Kidney Int* 47:1336–1345, 1995
43. Zager RA, Burkhart KM, Johnson ACM, Sacks B: Increased proximal tubular cholesterol content: Implications for cell injury and "acquired cytoresistance." *Kidney Int* 56:1788–1797, 1999
44. Honda N, Hishida A, Ikuma K, Yonemura K: Acquired resistance to acute renal failure. *Kidney Int* 31:1233–1238, 1987
45. Zager RA, Iwata M, Burkhart KM, Schimpf BA: Post ischemic acute renal failure protects proximal tubules from O_2 deprivation, possibly by inducing uremia. *Kidney Int* 47:1760–1768, 1994
46. Van Wyck D: Error, not iron, causes cytotoxicity. *Am J Kidney Dis* 40:1109–1110, 2002
47. Zager RA, Schimpf BA, Gmur DJ: Physiologic pH: Effect on posthypoxic proximal tubular injury. *Circ Res* 72:837–846, 1993
48. Agarwal R, Vasavada N, Sachs NG, *et al*: Oxidative stress and renal injury with intravenous iron in patients with chronic kidney disease. *Kidney Int* (in press)