PARENTERAL IRON FORMULATIONS DIFFERENTIALLY AFFECT
MCP-1, HO-1, AND NGAL GENE EXPRESSION
AND RENAL RESPONSES TO INJURY

Ali CM Johnson, BS
Kirsten Becker, BS
Richard A. Zager, MD

From: The Department of Medicine, University of Washington, and the Clinical Division, Fred Hutchinson Cancer Research Center, Seattle, WA

Running title: Parenteral Fe effects on kidney

Address correspondence to:
Richard A. Zager, MD
Fred Hutchinson Cancer Research Center
1100 Fairview Ave N; Room D2-190
Seattle, WA 98109
Email: dzager@fhcrc.org
Fax: 206 667-6519

Key words: ferumoxytol, Fe gluconate, Fe sucrose, heme oxygenase 1, NGAL, MCP-1

Grant support: This work was supported by research grants from the National Institutes of Health DK38432 and AMAG Pharmaceuticals.
ABSTRACT

Despite their pro-oxidant effects, ferric iron compounds are routinely administered to patients with renal disease to correct Fe deficiency. This study assessed relative degrees to which three clinically employed Fe formulations (Fe sucrose, FeS; Fe gluconate; FeG; ferumoxytol; FMX) impact renal redox-sensitive signaling, cytotoxicity, and responses to superimposed stress (endotoxin; glycerol induced ARF). Cultured human proximal tubule (HK-2) cells, isolated proximal tubule segments (PTS), or mice were exposed to variable, but equal, amounts of FeS, FeG, or FMX. Oxidant stimulated signaling was assessed by heme oxygenase 1 (HO-1) or MCP-1 mRNA induction. Cell injury was gauged by MTT assay (HK-2 cells), %LDH release (PTS), or renal cortical NGAL protein / mRNA levels. Endotoxin sensitivity and ARF severity were assessed by TNF-α and BUN concentrations, respectively. FeS and FeG induced lethal cell injury (in HK-2 cells, PTS), increased HO-1 and MCP-1 mRNAs (HK-2 cells; in vivo), and markedly raised plasma (~10x), and renal cortical (~3x) NGAL protein levels. Both renal and extra-renal (e.g., hepatic) NGAL production likely contributed to these results, based on assessments of tissue and HK-2 cell NGAL mRNA. FeS pre-treatment exacerbated endotoxemia. However, it conferred marked protection against the glycerol model of ARF (halving azotemia). FMX appeared to be “bioneutral”, as it exerted none of the above noted FeS / FeG effects. We conclude that: 1) parenteral iron formulations that stimulate redox signaling can evoke cyto/nephrotoxicity; 2) secondary adaptive responses to this injury (e.g., HO-1 / NGAL induction) can initiate a renal tubular cytoresistant state; this suggests a potential new clinical application for IV Fe therapy; and 3) FMX is “bioneutral”, regarding these responses. The clinical implication(s) of the latter, vis a vis the treatment of Fe deficiency in renal disease patients, remains to be defined.
INTRODUCTION

Patients with end stage renal disease are typically Fe deficient because of poor gut Fe absorption and ongoing blood losses (e.g., due to dialysis, frequent diagnostic phlebotomies; ref. 11,15). Because of this, intravenous (IV) Fe therapy, combined with erythropoietin stimulating agents (ESAs), has become a mainstay in the management of dialysis patients (9). Currently employed IV Fe formulations consist of a ferric iron core surrounded by various carbohydrate “shields”. The latter are designed to mitigate free Fe$^{3+}$ exposure, and hence, Fe mediated oxidative stress. The degree to which these various carbohydrate shields accomplish this goal, and thus, mitigate potential adverse clinical reactions, remains a subject of debate (1,44,45). So called “first generation” Fe compounds, the Fe dextrans (FeD), were associated with a risk of anaphylaxis (3, 14). Despite the subsequent release of improved, ‘low molecular weight’ FeD formulations (e.g. “InFed”), ongoing concerns of potential life threatening allergic reactions continue to limit FeD use. Currently, so called “second generation” agents, most notably Fe sucrose (FeS) and Fe gluconate (FeG), are most widely employed (2,9,28,34). While reported to have a low risk of allergic reactions, both FeS and FeG still possess potent pro-oxidant effects (44,45). Thus, there is no completely ‘non toxic’ IV iron formulation (4).

Ferumoxytol (FMX) is an Fe oxide nanoparticle which is coated with a polyglucose sorbitol carboxymethylether (6, 21, 30, 35, 36). It was initially designed for use as an MRI-angiographic contrast agent, and as such, it was formulated to permit rapid IV administration of large Fe dosages (10). It was subsequently demonstrated that when so administered, FMX can effectively replete Fe stores in hemodialysis patients. One potential reason for FMX’s ‘relative safety’, despite high dose bolus injection, is a reportedly low level of free (‘catalytic’) Fe, compared to other agents (FeS, FeG) (18, 20). However, this assumption is based solely on the results of in vitro testing with the bleomycin assay (18,20). This measures the ability of bleomycin to complex free Fe, and then generate ‘thiobarbituric acid reactive substances’ (TBARs) from exogenously added DNA. Of note, the Fe - bleomycin assay is fraught with
difficulties regarding specificity, reproducibility, and extreme sensitivity, potentially clouding data interpretation. Hence, it remains unclear as to whether FMX is, indeed, less pro-oxidant than current mainstay IV Fe formulations, and whether in vivo correlates of potential in vitro toxicity exist.

The purpose of the present study was to gain insights into this issue, and to better define potential IV Fe effects on the kidney. To this end, studies were undertaken in: i) human derived cultured HK-2 proximal tubular cells; ii) isolated mouse proximal tubule segments; and iii) CD-1 mice. Direct Fe toxicity (BUN, LDH release, MTT assay, NGAL formation), redox sensitive cytokine induction (TNF-α, MCP-1; ref. 19, 27), Fe sensitive heme oxygenase-1 (HO-1) expression (26), and the ability of different Fe formulations to alter superimposed renal injury responses (induced by either endotoxin or glycerol-induced acute renal failure) were assessed.

**METHODS**

A) Cell culture experiments.

*Cell viability following Fe exposure.* All cell culture experiments were performed with HK-2 cells, an immortalized proximal tubule cell line derived from normal human kidney (31). They were cultured with keratinocyte serum free medium (K-SFM) supplemented with pituitary extract, glutamine, and penicillin / streptomycin, as previously described in detail (31). For experimentation, the cells were trypsinized and seeded into 24 well Costar plates. Six hr later, wells in any given culture plate were exposed to either 500 μg/mL or 1000 μg/mL of elemental iron (Fe³⁺) in the form of either Fe sucrose (FeS; Venofer; American Regent Pharmaceuticals; Shirley NY), Fe gluconate (FeG; Watson Pharmaceuticals; Morristown, NJ), or ferumoxytol (FMX; AMAG Pharmaceuticals, Lexington, MA). Co-incubated cells maintained under control culture conditions served as controls. After 18 hr incubations, viable cell numbers were assessed by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), as previously described (31). Viable cells transport MTT into their mitochondria, the compound is
then reduced to formazin (purple color), and the latter is quantified colorometrically. Thus, the amount of color formed corresponds to viable cell numbers. Thus, the MTT assay is widely used for cytotoxicity assessments of pharmacologic and chemical agents (17, 25). These experiments were repeated on 2 occasions (n, 8 determinations per group). The values were presented as a % of the MTT uptake that was observed in control incubated cells.

*HK-2 cell oxidant responses:* MCP-1 and HO-1 are highly redox sensitive genes, and with an oxidant challenge, increases in their respective mRNAs result (9, 26, 27). Hence, we tested the relative abilities of FeG, FeS and FMX to alter MCP-1 and HO-1 mRNA levels in HK-2 cells. To this end, HK-2 cells were incubated overnight under control conditions or with 100 μg/mL FeS or FMX. After 18 hr incubations, the cells underwent total RNA extraction and HO-1 mRNA, MCP-1 mRNA, and GAPDH mRNAs were determined by competitive RT-PCR as previously described (40,43-45). MCP-1 and HO-1 mRNA levels were expressed as a ratio to simultaneously obtained GAPDH levels (serving as a “housekeeping” gene; n, 4 determinations for each mRNA).

**Dose titration of FeS concentrations and HK-2 cell HO-1 mRNA responses.** The following experiment was undertaken to ascertain whether HK-2 cell responses to the Fe compounds can be observed at clinically relevant Fe concentrations. Based on current dosing regimens and a normal intravascular volume, an IV FeS dose of 100 mg would be expected to yield a plasma concentration of ~25-35 μg/mL. HK-2 cells were grown either under control conditions or with the addition of either 25, 10, 5, or 1 μg/mL of FeS for 3 days. Additional wells of cells were exposed to 25 μg/mL of FeG or FMX x 3 days. At the completion of these incubations, oxidant stress was assessed by measuring HK-2 cell HO-1 mRNA levels.

**B) Mouse isolated proximal tubule segment (PTS) experiments.**

The following experiment assessed whether the cytotoxicity results observed in HK-2 cells with MTT assay could be recapitulated in a second in vitro system. To this end, isolated
mouse proximal tubule segments were studied. Four sets of mouse proximal tubule segments (PTS) were isolated from male CD-1 mice (~25-30 grams; Charles River Laboratories, Wilmington, MA) as previously described (40, 45). In brief, the mice were anesthetized with pentobarbital (~2 mg IP), the kidneys were immediately removed through a midline abdominal incision, and the cortices were recovered by dissection with a razor blade on an iced plate. The cortical tissues were minced with a razor blade, digested with collagenase, passed through a stainless steel sieve, and then pelleted by centrifugation (4°C). Viable PTS were then recovered by centrifugation through 32% Percoll (Pharmacia; Piscataway, NJ). After multiple washings in iced buffer, the PTS were suspended (~2-4 mg PTS protein/mL) in experimentation buffer (in mmol/L: NaCl, 100; KCl, 2.1; NaHCO\textsubscript{3} 25; KH\textsubscript{2}PO\textsubscript{4}, 2.4; MgSO\textsubscript{4} 1.2; MgCl\textsubscript{2}, 1.2; CaCl\textsubscript{2} 1.2; glucose 5; alanine 1; Na lactate 4; Na butyrate 10; 36 Kd dextran, 0.6%) and gassed with 95% O\textsubscript{2} / 5% CO\textsubscript{2} ; final pH, 7.4). Finally, they were re-warmed to 37°C in a heated shaking water bath over 15 min and then used for experimentation. Four PTS preparations were each divided into 4 equal aliquots (1.25 mL of PTS suspension placed into 10 mL Erlenmeyer flasks) and incubated for 60 min at 37°C either under control conditions (buffer only) or with 2 mg/mL of Fe\textsuperscript{3+} in the form of FeS, FeG, or FMX. After completing the 60 min incubations, cytotoxicity was assessed by determining the % tubular lactate dehydrogenase (LDH) release (44,45).

**C) In vivo assessments of MCP-1 and HO-1 expression.**

**3 hr post IV Fe administration: MCP-1 and HO-1 assessments.** CD-1 mice were placed into restraining tubes and subjected to a tail vein injection of either FeS, FeG, or FMX (2 mg elemental Fe). Control mice received 100 μL tail vein saline injections. Three hrs later, the mice were anesthetized with pentobarbital, the abdominal wall was opened, a plasma sample was obtained from the inferior vena cava, and the kidneys were resected. The plasma samples were used to measure MCP-1 by ELISA (BD Biosciences, San Jose, CA). The kidneys were cooled to 4°C, the cortices were dissected, and total RNA was extracted (RNaseasy Kit; Qiagen). As an index of renal cortical oxidative stress, the samples were analyzed for MCP-1 and HO-1.
mRNAs by RT-PCR, as previously described (30,39, 43-45). The results were expressed as ratios to simultaneously obtained GAPDH product (n, 5,4,6, and 10 for FeS, FeG, FMX and control mice, respectively).

18 hr post IV Fe administration: MCP-1 and HO-1 assessments: The above experiment was repeated, except that the plasma and renal cortical samples were obtained at a more delayed time point: 18 hrs post Fe or vehicle injections. Six mice were used for each Fe treatment and the results were contrasted to those observed in 6 controls. Terminal BUN concentrations were also assessed.

D) NGAL assessments.

Renal cortex: NGAL is widely recognized as a marker of subclinical or overt renal tubular injury (8, 13, 23). To further assess whether the Fe preparations have an intrinsic cytotoxic potential, renal cortical NGAL levels and its mRNA were assessed at different time points post Fe injections. Mice were injected either with FeS (n, 8), FeG (n, 8), FMX (n, 8) or saline (n, 14) via the tail vein as noted above. Either 3 hrs or 18 hrs later (half of the mice in each group at each time point), they were anesthetized, the kidneys were removed, and the cortices were subjected to protein extraction. NGAL levels were assessed using a commercially available assay (R and D Systems). Values were expressed as pg/mg tissue protein extract. In addition, the 18 hr samples were also extracted for total RNA to assess whether observed NGAL protein changes (see Results) were paralleled by changes in NGAL mRNA. The primers used for NAGL mRNA are presented in Table 1.

Plasma NGAL concentrations and extra-renal NGAL mRNA expression. The following experiment was undertaken to ascertain whether renal cortical NGAL concentrations may have been impacted by high circulating NGAL concentrations, and if so, whether extra-renal organs respond to IV Fe injection with an increase in NGAL mRNA. Mice were injected via the tail vein with 2 mg of Fe in the form of FeS, FeG, or FMX or vehicle (6 mice per group). After 18 hrs,
they were anesthetized, plasma was obtained from the inferior vena cava. In addition, 3 mice per group had pieces of liver, spleen, and lung excised for assessment of NGAL mRNA.

**Assessment of direct Fe effects on proximal tubule NGAL induction.** HK-2 cells were exposed to 25 μg/mL of either FeS, FeG, or FMX for 3 days. Co-incubated wells of cells served as controls (n 6 in each group). The cells were extracted for RNA and assayed for NGAL and GAPDH mRNA, using the primer pairs listed in table 1.

Because FeS and FeG were found to suppress NGAL mRNA levels (see Results), we next assessed whether this might have been secondary to an Fe mediated up-regulation of HO-1 (i.e., conferring cytoprotection against Fe, and thus, blunting NGAL induction). Additional HK-2 cells were cultured with 25 μg/mL FeS or under control incubations as noted above. After 48 hrs, half of the wells of cells were exposed to the HO-1 inhibitor tin protoporphyrin x 24 hrs (SnPP; 100 μM; n, 4 per group). The impact of the SnPP on NGAL mRNA levels was then assessed. HO-1 mRNA was also determined to confirm that effective HO-1 inhibition had occurred (i.e., to document the expected compensatory increase in HO-1 mRNA).

**Fe “pre-conditioning”: effect on glycerol induced rhabdomyolysis ARF.** It has previously been documented that Fe induced oxidative stress, e.g., as evoked by myoglobin, can confer striking protection against episodes of oxidant mediated acute renal failure (e.g., as induced by glycerol or cisplatin ARF models; ref. 26, 33). Because FeS, but not FMX, evoked oxidant stress, it was hypothesized that pre-treatment with FeS, but not with FMX, might protect against subsequent ARF. To test this hypothesis, mice were injected with 2 mg of either FeS (n, 7) or FMX (n, 5) or control tail vein injections (n, 11), as noted above. Approximately 18 hrs later, the mice were lightly anesthetized with isoflurane, and subjected to intramuscular hypertonic glycerol injection (8 mL/Kg; 50% solution; administered in equally divided doses in upper hind limbs). At 18 hrs post glycerol injection, the mice were deeply anesthetized with pentobarbital, a plasma sample
was drawn from the inferior vena cava, and the kidneys were removed. The plasma samples were assayed for BUN and the renal cortices were assayed for HO-1 and MCP-1 mRNAs.

**Fe pre-conditioning: effect on Gram negative bacterial endotoxin (lipopolysaccharide; LPS) mediated TNF-α generation.** We previously demonstrated that prior IV FeS or FeG treatment sets the stage for an exaggerated TNF-α response to LPS injection (41, 45). The following experiment assessed whether this phenomenon is also evoked by FMX- induced Fe loading. Mice received tail vein injections of either FeS (n,4), FeG (n,4) FMX (n,4), or saline (n,8). Approximately 18 hrs later, each of the mice received an intraperitoneal injection of 10 mg/Kg E. coli LPS (0111:B4; L-2630; Sigma Chemicals, St Louis, MO; stock solution, 4 mg/mL saline). Two hrs post LPS injection, the mice were anesthetized, and plasma and renal cortical samples were obtained. The plasma samples were assayed for TNF-α (ELISA; R&D Systems). Renal cortical samples were subjected to RT-PCR to quantify TNF-α mRNA levels (41, 45).

**Animal utilization; Calculations and Statistics.**

All values are presented as means ± 1 SEM. Statistical comparisons were performed by unpaired Student’s t test. If multiple comparisons were made, the Bonferroni correction was applied. Mouse protocols were approved by the Fred Hutchinson Cancer Research Center's IACUC committee.

**RESULTS**

**Cell culture experiments.**

**Cell viability:** As shown in Fig. 1, at a dose of 500 μg/mL, only FeS caused a significant decrease in MTT uptake (36% of the values observed in the control cells). At the 1000 μg/mL concentration, each of the Fe preparations decreased MTT uptake, with the degree of suppression being FeS >> FeG > FMX.
**HO-1 and MCP-1 mRNA induction:** As shown in Fig. 2, FeS induced marked increases in HK-2 cell HO-1 and MCP-1 mRNAs. Conversely, FMX evoked no increase in either mRNA, consistent with a lack of significant oxidative stress.

**HO-1 mRNA dose – response relationship.** As shown in Fig. 3, a steep dose response relationship between FeS dose and HK-2 cell HO-1 mRNA induction was observed. Even at a 1μg/mL FeS dose x 3 days, a significant increase in HO-1 mRNA was observed. The 25 μg/mL “clinically relevant” FeG dose also significantly increased HO-1 mRNA. Conversely, 25 μg/mL of FMX did not impact HO-1 mRNA levels (NS vs. controls).

**Isolated proximal tubule segment experiments.**

As shown in Fig. 4, FeS caused the greatest toxicity of the three tested Fe preparations (% LDH release, 41%). FeG evoked mild, but statistically significant, cytotoxicity. Conversely, FMX had no overt cytotoxic effect.

**In vivo mouse experiments.**

**3 hr post Fe injection: MCP-1 and HO-1 assessments.** As shown in the Fig. 5, left, within 3 hrs of FeS or FeG injection, only slight, and non significant, increases in renal cortical MCP-1 mRNA were observed. However, ~3 fold increases in circulating MCP-1 protein were noted (Fig. 5, right), consistent with either increased renal or extra-renal MCP-1 production. Conversely, FMX evoked no increase in serum MCP-1 levels. If anything, FMX mildly suppressed renal cortical MCP-1 mRNA values, compared to the FeS and FeG treatment groups (p<0.05). As shown in Fig. 6, FeS and FeG each induced dramatic increases in renal cortical HO-1 mRNA levels, whereas FMX did not impact this marker of Fe mediated oxidative stress.

**18 hr post Fe injection: MCP-1 assessments.** By 18 hrs post FeS or FeG injection, significant increases in renal cortical MCP-1 mRNA values were observed, vs. control values (Fig. 7, left panel). These increases corresponded with striking increases in plasma MCP-1 levels (Fig. 7,
right panel). Conversely, by 18 hrs post FMX injection, no significant changes in either MCP-1 mRNA or in plasma MCP-1 protein levels were observed. None of the Fe formulations significantly altered BUN concentrations (<28 mg/dL for Fe and control groups).

**NGAL assessments.**

- **Renal cortex:** As shown in Fig. 8, FeG injection caused ~2 fold and ~5 fold increases in renal cortical NGAL concentrations at 3 hr and 18 hrs respectively (compared to control values). FeS caused a 4 fold NGAL increase, but this change was only observed at the 18 hr time point. In contrast, FMX failed to evoke any renal cortical NGAL increase at either the 3 hr or 18 hr time points. Consistent with these results, both FeS and FeG significantly increased renal cortical NGAL mRNA, whereas FMX did not alter renal NGAL mRNA levels.

- **Plasma NGAL concentrations and extra-renal NGAL mRNA levels:** As shown in Fig. 9, left, FeS injection and FeG induced 8-20 fold increases in plasma NGAL levels (vs. control plasma samples). Conversely, FMX did not alter plasma NGAL concentrations. The high circulating NGAL levels likely reflected, at least in part, extra-renal NGAL production, given that FeS and FeG, but not FMX, markedly increased hepatic NGAL mRNA levels (Fig. 9, right). In contrast, none of the Fe compounds increased splenic or pulmonary mRNA levels (data not shown).

- **Direct Fe effects on proximal tubule NGAL induction ± HO-1 inhibition.** As shown in Fig. 10, FeS and FeG markedly suppressed HK-2 cell NGAL mRNA (to ~20% of control values). This was not due to a Fe mediated increase in HO-1 activity because the HO-1 inhibitor, SnPP, did not prevent the Fe mediated NGAL mRNA suppression. That SnPP had effectively inhibited HO-1 in these experiments was indicated by the fact that it caused an approximate 8 fold increase in HO-1 mRNA (Fig. 10 right). In striking contrast to FeS and FeG, FMX did not alter HK-2 NGAL mRNA levels.

**Fe effects on superimposed in vivo renal injury**

- **Fe pre-conditioning: effect on glycerol induced rhabdomyolysis ARF.** As shown in Fig. 11, left panel, FeS pre-treatment significantly attenuated the severity of glycerol-induced ARF, as
denoted by a 50-60% reduction in BUN concentrations. A correlate of this protection was a 50-
60% reduction in glycerol-induced HO-1 mRNA elevations (Fig. 11, right panel). A significant
correlation existed between the BUN and MCP-1 mRNA concentrations (glycerol alone, 1.1 ±0.4; FeS 0.52 ± 0.15; r, 0.56; p<0.05), also consistent with a FeS induced diminution in
glycerol-induced oxidative stress. In contrast, FMX pre-treatment did not attenuate the severity
of glycerol induced ARF, as assessed by BUN concentrations or HO-1 mRNA (Fig. 11).

Impact of Fe loading on LPS mediated TNF-α induction. None of the Fe formulations, by
themselves, increased renal cortical TNF-α mRNA levels (controls, 0.3±0.1; FeS, 0.5±0.2; FeG,
0.4±0.1; FMX 0.4 ±0.1) or plasma TNF-α (undetectable levels). However, FeS and FeG each
sensitized to LPS driven TNFα production, as reflected by preferential increases in plasma
TNF-α concentrations and renal cortical TNF-α mRNA levels (p<0.001<0.005), compared to
non Fe treated LPS injected controls. Conversely, prior FMX administration did not alter LPS
responsiveness, as gauged by renal TNFα mRNA or circulating TNF-α levels (Fig.12).

DISCUSSION

It has been firmly established that IV Fe therapy effectively repletes Fe stores and enhances
erthropoietin (Epo)-mediated red blood cell production in patients with chronic renal disease.
However, concerns persist that, despite its overall beneficial effects, IV Fe administration might
also exert adverse effects. For example, in three large clinical trials that involved pre-dialysis or
dialysis patients, aggressive IV Fe / Epo use, targeting hemoglobin concentrations of ≥13
gm/dL, was associated with increased cardiovascular complications and/or mortality, compared
to less aggressive therapy (2, 28, 34). Because IV iron and Epo are typically used together, it
has not been possible to clearly dissect out whether these increased complications were directly
caused by Epo alone, Fe alone, or the combination of these two agents. That currently used IV
Fe formulations have potent pro-oxidants effects (e.g., ref. 44,45), and that oxidant stress is well
known to evoke cardiovascular injury and systemic inflammation (5, 12, 22, 29, 37), provide a
theoretical basis for assuming that IV Fe may have played a role. However, it is notable that in the TREAT study, no direct relationship between IV Fe dose and cardiovascular complications appeared to exist (28).

Because of structural differences in currently employed Fe formulations (7), the degrees to which they induce oxidant stress may vary. Indeed, the results of our previous study (45) which compared FeS, FeG, and FeD supports this view, given that Fe mediated cytotoxicity greatly varied amongst three test agents (FeS>> FeG> FeD). Since the time of that initial study, FMX has gained FDA approval and has entered into clinical use. Hence, we sought to determine FMX’s relative cytotoxic and pro-oxidant potentials, compared to the two most widely used parenteral Fe formulations (FeS, FeG). To gain initial insights, the ability of these three compounds to induce lethal cell injury in cultured HK-2 cells was assessed. As shown in Fig. 1, the rank order of toxicity, as assessed by MTT uptake, was FeS >> FeG > FMX. It is noteworthy that a reduction in MTT uptake indicates cytotoxicity, in general, and not Fe mediated oxidative stress, per se. Hence, to more directly assess this latter issue, the impact of FeS and FMX on HK-2 cell expression of two redox sensitive genes, HO-1 and MCP-1, was examined. As shown in Fig. 2, FeS increased both HO-1 and MCP-1 mRNAs. Conversely, FMX was without effect. Thus, these two sets of HK-2 cell results support the initial hypothesis: that differing Fe “shields”, as used in parenteral Fe formulations, can, indeed, have a striking impact on Fe’s cytotoxic and pro-oxidant effects.

Unlike in vivo proximal tubules which are almost completely dependent on mitochondrial (aerobic) ATP production, cultured proximal tubule (including HK-2) cells have substantial glycolytic capacity (16). Because mitochondrial oxygen consumption is the dominant source of oxygen free radical generation in renal cortex (38), results obtained with glycolytic cells may not be directly relevant to the in vivo state. Thus, to confirm differential toxicity within proximal tubule epithelium, this issue was addressed using almost strictly aerobic proximal tubules, isolated from normal mice. As depicted in Fig. 3, both FeS, and to a lesser degree, FeG,
exerted direct cytotoxicity, as assessed by LDH release. Conversely, FMX had no overt cytotoxic effect. Thus, these data were entirely consistent with the HK-2 cell results.

Given the above in vitro observations, we next sought evidence of Fe mediated cytotoxicity in the in vivo state. Of note, none of the three test Fe formulations evoked overt renal injury, as denoted by normal post infusion BUN concentrations (<28 mg/dL). Hence, a more sensitive marker of cytotoxicity was sought. Over the past 8 years, neutrophil gelatinase-associated lipoprotein (NGAL) has been recognized as having utility in this regard (8, 13, 23, 33). It is up-regulated in response to diverse forms of cellular stress, and like heat shock proteins, it may confer a survival benefit upon sublethally damaged cells (24, 32). As shown in Fig. 8, FeG administration evoked an early (3 hr) and progressive increase in renal cortical NGAL levels, such that by 18 hrs post injection, an 8 fold increase in NGAL protein, and a 4 fold increase in its mRNA, were observed. FeS largely recapitulated these FeG results. Conversely, FMX did not evoke any increase in renal cortical NGAL protein or mRNA. Thus, these in vivo results were entirely consistent with the HK-2 cell and isolated tubule data: i.e., that FMX appears to have a low cytotoxic potential, compared to the FeS and FeG formulations.

It is important to note that increases in renal cortical NGAL mRNA and protein are not necessarily mechanistically linked. For example, it has recently been demonstrated that injury-induced renal NGAL mRNA elevations occur predominantly within the loop of Henle and collecting duct (32). This is a seeming paradox, given that the proximal tubule is the dominant site of both nephrotoxic and ischemic renal damage. Thus, it is now hypothesized that the bulk of injury-induced renal cortical NGAL protein accumulation arises from extra-renal NGAL production, followed by glomerular filtration, and megalin / 24p3R receptor-mediated proximal tubule update (32). Given these considerations, we assessed the impact of the IV Fe formulations on plasma NGAL levels, and tested whether the Fe preparations impact extra-renal, as well as proximal tubule specific (HK-2 cell), NGAL gene induction. As shown in Fig. 9, FeS and FeG markedly increased plasma NGAL levels, and corresponding increases in extra-
renal NGAL mRNA (e.g., in liver) were observed. Conversely, FeS and FeG induced profound suppressions of proximal tubule (HK-2) cell NGAL mRNA production. Thus, when these in vivo and in vitro results are viewed together, they strongly suggest that the FeS / FeG- mediated renal cortical NGAL protein increases were derived, at least in part, from the systemic circulation, and not from proximal tubule cells. The reason why FeG and FeS suppressed HK-2 cell NGAL mRNA remains unknown. Of note, it cannot simply be explained by an Fe mediated concomitant increase in cytoprotective HO-1 expression, given that HO-1 inhibition (with SnPP) did not reverse Fe’s suppressive effect. NGAL is currently viewed as an intracellular Fe delivery system. Thus, it seems possible that when proximal tubules become Fe overloaded (e.g., with IV Fe therapy), a compensatory down-regulation of the NGAL-Fe delivery system results. In striking contrast to FeG and FeS, FMX neither increased plasma NGAL nor altered extra-renal or proximal tubular cell NGAL mRNA. This additionally underscores fundamental differences in biologic activity of FMX, compared to the two other IV Fe formulations.

Given the apparent absence of FMX toxicity in each of the above experiments, we hypothesized that FMX would exert a lesser effect on in vivo HO-1 and MCP-1 expression than FeS and FeG (39, 41, 42-45). Indeed, this was the case. Whereas FeS or FeG each increased plasma MCP-1 and renal cortical MCP-1 mRNA levels, FMX had no effect. Furthermore, unlike FeS and FeG, FMX failed to raise renal cortical HO-1 mRNA. Thus, these data are again entirely consistent with the hypothesis that FMX has a lower pro-oxidant potential than the other two test Fe formulations. The degree to which the plasma MCP-1 elevations reflected intra-renal vs. extra-renal production remains unknown.

In each of the above studies, direct Fe effects were assessed. However, it should also be noted that IV Fe formulations can impact tissue damage by altering superimposed injury responses. As one example, if mice are pre-treated with either FeS or FeG, increased susceptibility to subsequent Gram negative sepsis or LPS mediated cytokine generation results (41, 43-45). Thus, we next questioned whether FMX might lack this “LPS- sensitizing” effect.
As shown in Fig. 12, this was the case: whereas both FeS and FeG pre-treatment sensitized to LPS driven TNF-α production, FMX did not alter this LPS response. A second pathway by which Fe may impact subsequent tissue injury is by evoking cytoprotective stress proteins which then protect against ‘downstream’ tissue damage. One of the best examples of this principle, so called “acquired resistance”, is Fe mediated HO-1 production which then protects against subsequent oxidant attack (25, 26). Recently, it has been suggested that NGAL also possesses cytoprotective effects (24). Because FeS, but not FMX, increased renal cortical HO-1 and NGAL expression, we hypothesized that the former, but not the latter, would protect against the glycerol model of rhabdomyolysis induced ARF. As shown in Fig. 11, FeS “pre-conditioning” did indeed, attenuate glycerol induced renal injury, as denoted by ~50% reductions in both azotemia and glycerol-induced HO-1 induction. Conversely, FMX pre-treatment conferred no cytoprotective effect. These data raise a compelling clinical question: might it be possible to use pro-oxidant Fe compounds to ‘pre-condition” patients, and thus, confer renal protection against procedures or treatments that have a high risk of inducing ARF (e.g., cardiovascular surgery, radioccontrast administration, or cisplatin therapy)? If so, based on the present findings, it would appear that either FeS or FeG would have greater utility in this regard compared to compounds with lesser pro-oxidant effects (e.g., FMX).

Finally, for a number of reasons, we would like to stress the present results cannot be readily extrapolated to the clinical arena. First, in this study, we used identical amounts of, and time exposures to, FeS, FeG, and FMX in order to assess relative degrees of pro-oxidant and cytotoxic effects. However, in clinical practice, marked differences in Fe dosing regimens exist. For example, FMX is typically administered as a single 510 mg bolus. Conversely, FeS and FeG are infused more slowly (e.g. 30-60 min), in lower amounts (~100-125 mg per treatment), but with repetitive dosing. Whether these dosing differences ultimately impact potential parenteral Fe effects remains unknown. Second, although FeS and FeG have greater intrinsic pro-oxidant potentials than FMX, it is intriguing to speculate that following their initial
administration, FeS and FeG up-regulate HO-1 and NGAL which, via the cytoprotective properties, might serve to negate subsequent adverse effects. This may be particularly germane in light of the fact that IV Fe formulations are given on a long term basis, rather than acutely as done in this study. Third, it is critical to note that the present study did not assess the risk of acute allergic reactions to IV Fe compounds, and the latter represent the major recognized complication of IV Fe therapy; and fourth, the present study, for the most part, utilized Fe dosages that were in great excess to those that are clinically deployed. This general approach is by no means unique to this study, since virtually all rodent studies of nephrotoxicity require extremely high drug concentrations in order to model clinically observable toxic effects. However, in this regard, it is notable that even at a dose of 1 μg/ml FeS (~5% of plasma concentrations following IV injection in patients), HK-2 cell HO-1 mRNA induction was observed. That Agarwal et al (1) noted differential Fe compound toxicity in humans, using proteinuria as a endpoint, further suggests that clinical correlates to the current study may well exist. Hopefully, the present results will stimulate future trials to evaluate this possibility in the clinical arena, particularly in light of the fact that parental Fe administration is increasingly being administered to patients with renal disease.
REFERENCES


Table 1. Primers for RT-PCR

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer Sequences</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human NGAL</td>
<td>5'-TCT CAG AGA AGA CAA AGA CCC GCA-3' (forward) 5'-AGT TCC GAA GTC AGC TCC TTG GTT-3' (reverse)</td>
<td>321 bp</td>
</tr>
<tr>
<td>Mouse NGAL</td>
<td>5'-AAC ATT TGT TCC AAG CTC CAG GGC-3' (forward) 5'-CAA AGC GGG TGA AAC GTT CCT TCA-3' (reverse)</td>
<td>224 bp</td>
</tr>
</tbody>
</table>

Table 1 legend. The primers used for quantitating mouse NGAL mRNA and human NGAL mRNA (HK-2 cells) are presented.
**FIGURE LEGENDS**

**Figure 1. Impact of ferumoxytol (FMX), Fe gluconate (FeG) and Fe sucrose (FeS) on HK-2 cell viability, as assessed by MTT uptake.** HK-2 cells were incubated x 18 hrs with either 500 or 1000 μg/mL of the three test Fe compounds and then the number of viable cells remaining were assessed by MTT uptake (uptake directly correlates with the number of viable cells). The values obtained are expressed as a % of that observed in control (C) incubated cells. At 500 μg/mL, only FeS caused a significant decrease in viable cells. At 1000 μg/mL, all three drugs decreased viable cell numbers in the rank order of FeS>> FeG > FMX.

**Figure 2. Fe compound effects on HK-2 cell HO-1 and MCP-1 mRNA levels.** HK-2 cells were exposed to a subtoxic dose of FeS (100 μg/mL) or to an equal dose of FMX. Eighteen hrs later, the cell RNA extracts were assayed for HO-1 and MCP-1 mRNA. FeS induced marked increases in HO-1 and MCP-1 mRNAs; conversely, FMX had no effect.

**Figure 3. Dose – response relationship between FeS and HK-2 cell HO-1 mRNA.** HK-2 cells were exposed to 0 - 25 μg/mL of FeS x 3 days, followed by HO-1 mRNA assessments. A steep dose - response relationship was observed, with a statistically significant effect being observed at the lowest test concentration (1 μg/mL). A 3 day exposure to 25 μg/mL of FeG also raised HO-1 mRNA, whereas an equal amount of FMX was without effect.

**Figure 4. Fe compound cytotoxicity, as assessed in isolated mouse proximal tubule cells.** Proximal tubules were incubated for 60 min under control (C) conditions or with 2mg/mL FeS, FeG or FMX, followed by assessment of lethal cell injury as gauged by % LDH release. FeS and FeG evoked 4x and 2x increases in cell death, whereas FMX was without a discernible effect.

**Figure 5. Fe compound effects on in vivo MCP-1 induction, as assessed by renal cortical MCP-1 mRNA and plasma MCP-1 levels (3 hr assessments).** Mice were injected with either FeS, FeG, FMX or vehicle. Three hrs later, MCP-1 assessments were made. FeS and FeG
raised plasma MCP-1 levels (right panel). While significant elevations in renal cortical MCP-1 levels were not observed, a trend in that direction was observed (left panel). Conversely, FMX failed to increase plasma MCP-1 or renal cortical MCP-1 mRNA. The MCP-1 mRNA levels following FMX treatment were significantly lower than the values obtained with FeS and FeG treatment (p<0.05).

**Figure 6. Renal cortical HO-1 mRNA, as assessed 3 hrs post Fe or vehicle injections.**

Three hrs post Fe or control injections, HO-1 mRNA levels were assessed. FeS and FeG evoked 15-20 fold increases over control values. Conversely, FMX was without effect.

**Figure 7. Fe compound effects on in vivo MCP-1 induction, as assessed by renal cortical MCP-1 mRNA and plasma MCP-1 levels (18 hr assessments).** These experiments were conducted in an identical fashion to those presented in Fig. 5, except that the assessments were made 18 hrs post injections. FeS and FeG each raised renal cortical MCP-1 mRNA and plasma MCP-1 protein levels. Conversely, FMX was without effect.

**Figure 8. Renal cortical NGAL induction 3 and 18 hrs post Fe injections.** Both FeS and FeG injection raised renal cortical NGAL protein and NGAL mRNA levels. Conversely, FMX did not alter renal cortical NGAL expression, at either 3 or 18 hrs, whether assessed by protein or mRNA levels.

**Figure 9. Plasma NGAL levels 18 hrs post Fe injections and corresponding hepatic NGAL mRNA levels.** FeS and FeG each caused dramatic plasma NGAL increases, as assessed 18 hrs post injection. Corresponding increases in hepatic NGAL mRNA were observed, suggesting that increased hepatic NGAL synthesis contributed to the elevated plasma NGAL levels. Conversely, FMX did not alter plasma NGAL or hepatic NGAL mRNA levels.

**Figure 10. Fe effects on NGAL mRNA in HK-2 cells.** Both FeS and FeG caused profound suppressions of HK-2 cell mRNA levels. This could not be explained by an Fe mediated increase in HO-1 expression because HO-1 inhibition (with SnPP) did not prevent the Fe
mediated NGAL mRNA suppression. That SnPP effectively inhibited HO-1 in these experiments was indicated by the fact that it markedly increased HO-1 mRNA.

**Figure 11. Fe ‘preconditioning’ effects on subsequent glycerol- induced ARF.** Mice were pre-treated with either FeS, FMX or subjected to vehicle injection. Eighteen hrs later, each group of mice was subjected to glycerol injection to induce rhabdomyolysis ARF. FeS pre-treatment markedly reduced the severity of ARF, as assessed by BUN concentrations, and mitigated the glycerol- induced increases in HO-1 mRNA. Conversely, FMX pre-treatment did not alter the severity of glycerol induced ARF.

**Figure 12. Fe ‘preconditioning’ effects on subsequent LPS stimulated TNF-α production.** Mice were injected with one of the three test Fe preparations, or with saline (controls), followed 18 hrs later by LPS injection. Two hrs later, plasma TNF-α (protein) and renal cortical TNF-α mRNA levels were assessed. FeG and FeS each potentiated the observed LPS driven TNF-α increases, whereas FMX had no LPS “priming” effect.
HK-2 Cell MTT Uptake

% uptake (vs. control)

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>FMX</th>
<th>FeG</th>
<th>FeS</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 μg/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>FMX</th>
<th>FeG</th>
<th>FeS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000 μg/mL</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Isolated Mouse Proximal Tubule Segments

% LDH Release

C       FeS       FeG       FMX

<0.001

NS
Renal Cortical HO-1 mRNA

![Bar chart showing mRNA/GAPDH levels at 3 hrs post injection for different groups: C, FeS, FeG, FMX. The FeS group shows a significant increase compared to C and FMX, with NS indicating non-significance between FeG and FMX.](image)
Renal Cortical MCP-1 mRNA

<table>
<thead>
<tr>
<th>Group</th>
<th>MCP-1 mRNA / GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.25</td>
</tr>
<tr>
<td>FeS</td>
<td>0.75</td>
</tr>
<tr>
<td>FeG</td>
<td>0.75</td>
</tr>
<tr>
<td>FMX</td>
<td>NS</td>
</tr>
</tbody>
</table>

Plasma MCP-1

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma MCP-1 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>FeS</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FeG</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>FMX</td>
<td>300</td>
</tr>
</tbody>
</table>

18 hrs post injection
Renal NGAL (protein)

pg/ml protein

C  FeS  FeG  FMX

3 hrs

<0.002

NS

<0.01

<0.01

<0.02

<0.05

NS

Renal NGAL mRNA

C  FeS  FeG  FMX

18 hrs

NS

Renal NGAL (protein)

C  FeS  FeG  FMX

18 hrs
Plasma NGAL

Hepatic NGAL mRNA

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>FeS</th>
<th>FeG</th>
<th>FMX</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGAL</td>
<td>&lt;0.25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NGAL/GADH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>FeS</th>
<th>FeG</th>
<th>FMX</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGAL</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NGAL/GADH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NS indicates non-significance.
HK-2 cell NGAL mRNA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>mRNA/GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>7.5</td>
</tr>
<tr>
<td>FMX</td>
<td>12.5</td>
</tr>
<tr>
<td>FeG</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FeS</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FeS + SnPP</td>
<td>NS</td>
</tr>
</tbody>
</table>

HK-2 cell HO-1 mRNA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>mRNA/GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>2</td>
</tr>
<tr>
<td>SnPP</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
GLYCEROL

BUN (mg/dL)

Control FeS FMX

<0.005 NS

HO-1 mRNA / GAPDH

Control FeS FMX

<0.01 NS
Plasma TNF-α

Renal Cortex TNF-α mRNA

pg / mL

<0.001

<0.005

Overnight + 2 h LPS

C  FeS  FeG  FMX

NS

C  FeS  FeG  FMX

NS