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Non-transferrin bound iron, cytokine activation and intracellular reactive oxygen species generation in hemodialysis patients receiving intravenous iron dextran or iron sucrose

Amy Barton Pai · Todd Conner · Charles R. McQuade · Jonathan Olp · Paul Hicks

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Abstract Intravenous (IV) iron supplementation is widely used to support erythropoeisis in hemodialysis patients. IV iron products are associated with oxidative stress that has been measured principally by circulating biomarkers such as products of lipid peroxidation. The pro-oxidant effects of IV iron are presumed to be due at least in part, by free or non-transferrin bound iron (NTBI). However, the effects of IV iron on intracellular redox status and downstream effectors is not known. This prospective, crossover study compared cytokine activation, reactive oxygen species generation and oxidative stress after single IV doses of iron sucrose and iron dextran. This was a prospective, open-label, crossover study. Ten patients with end-stage renal disease (ESRD) on hemodialysis and four age and sex-matched healthy were assigned to receive 100 mg of each IV iron product over 5 min in random sequence with a 2 week washout between products. Subjects were fasted and fed a low iron diet in the General Clinical Research Center at the University of New Mexico. Serum and plasma samples for IL-1, IL-6, TNF-α and IL-10 and NTBI were obtained at baseline, 60 and 240 min after iron infusion. Peripheral blood mononuclear cells (PBMC) were isolated at the same time points and stained with fluorescent probes to identify intracellular reactive oxygen species and mitochondrial membrane potential ($\Delta\psi$m) by flow cytometry. Lipid peroxidation was assessed by plasma F_2 isoprostane concentration. Mean ± SEM maximum serum NTBI values were significantly higher among patients receiving IS compared to ID (2.59 ± 0.31 and 1.0 ± 0.36 μM, respectively, $P = 0.005$ IS vs. ID) Mean ± SEM NTBI area under the serum concentration–time curve (AUC) was 3-fold higher after IS versus ID (202 ± 53 vs. 74 ± 23 μM*min/l, $P = 0.04$) in ESRD patients, indicating increased exposure to NTBI. IV iron administration was associated with increased pro-inflammatory cytokines. Serum IL-6 concentrations increased most profoundly, with a 2.6 and 2.1 fold increase from baseline in ESRD patients given IS and ID, respectively ($P < 0.05$ compared to baseline). In healthy controls, serum IL-6 was undetectable at baseline and after IV iron administration. Most ESRD patients had increased intracellular ROS generation, however, there was no difference between ID and IS. Only one healthy control had increased ROS generation

A. B. Pai (✉)
Department of Pharmacy Practice, ANephRx-Albany Nephrology Pharmacy Group, Albany College of Pharmacy and Health Sciences, 106 New Scotland Avenue, Albany, NY 12208, USA
e-mail: amy.bartonpai@acphs.edu

T. Conner
VA Cooperative Studies Program, Albuquerque Veteran Affairs Medical Center, 1501 San Pedro NE, Albuquerque, NM 87108, USA

C. R. McQuade · J. Olp · P. Hicks
College of Pharmacy, University of New Mexico, 2502 Marble NE, Albuquerque, NM 87106, USA

post IV iron. All healthy controls experienced a loss of $\Delta\psi$m (100% with IS and 50% with ID). ESRD patients also had loss of $\Delta\psi$m with a nadir at 240 min. IS administration was associated with higher maximum serum NTBI concentrations compared to ID, however, the both compounds produced similar ROS generation and cytokine activation that was more pronounced among ESRD patients. The effect of IV iron-induced ROS production on pivotal signaling pathways needs to be explored.

Keywords Intravenous iron · Oxidative stress · Hemodialysis

Introduction

Intravenous (IV) iron is frequently administered to HD patients to support erythrocyte production by erythropoiesis stimulating agents (ESAs) (e.g. epoetin alfa). The commercially available IV iron compounds (iron dextran, iron sucrose, sodium ferric gluconate, and ferumoxytol) all consist of a ferric hydroxide core surrounded by a protective "carbohydrate shell" that is designed to prevent immediate dissolution of ferric iron (Danielson 2004). This should allow the compound to be properly metabolized by the reticuloendothelial system (RES). However, all IV iron products have the potential to have direct release of iron [non-transferrin bound iron (NTBI)] into plasma, a physicochemical characteristic that appears to be directly related to molecular weight, and drug clearance by the RES (Danielson 2004; Pai et al. 2010; Balakrishnan et al. 2009; Van Wyck et al. 2004). Single and multi-dose studies of IV iron have demonstrated significant increases in biomarkers of oxidative stress in HD patients indicating that IV iron can induce lipid peroxidation, DNA damage and increase inflammatory mediators (Van Campenhout et al. 2008; Kuo et al. 2008; Garcia-Fernandez et al. 2010). A comparative analysis of commercially available preparations has shown that in vitro that iron sucrose, sodium ferric gluconate and iron dextran induce oxidative stress but higher rates of cell death were apparent among iron sucrose, sodium ferric gluconate treated human proximal tubule cells (Zager et al. 2002). Conversely, the newly approved agent ferumoxytol, was not associated with increased oxidative stress or cell death and was considered "bioneutral" (Johnson et al. 2010). These data also suggest that small molecular weight compounds (e.g. iron sucrose) may be associated with greater toxicity. Smaller compounds may have different biologic presentation compared to the other agents that may permit increased cellular uptake, facilitating mitochondrial respiratory blockade, generation of reactive oxygen species and potential dysregulation of intracellular signaling. We have shown in chronic hemodialysis patients that the smaller molecular weight iron compounds (iron sucrose and sodium ferric gluconate) are associated with greater appearance of NTBI compared to the larger molecular weight compound, iron dextran (Pai et al. 2007). These data imply a potential increased risk in vivo with labile iron-carbohydrate compounds. However, given the numerous sources of oxidative stress in hemodialysis patients (Himmelfarb et al. 2002), plasma biomarkers may not be sensitive enough to detect acute changes in oxidative stress, nor do they fully illuminate intracellular changes such as reactive oxygen species generation that may affect principal signaling pathways. The purpose of this study was to investigate the effects of acute administration of low molecular weight (iron sucrose) and high molecular weight (iron dextran) intravenous iron compounds on cytokine activation, intracellular reactive oxygen species generation and lipid peroxidation in chronic hemodialysis patients compared with a group of healthy controls.

Subjects and methods

Patients and study design

This was a prospective, open-label, crossover study. The study was approved by the University of New Mexico Human Research Review Committee. Informed consent was obtained from all subjects prior to initiation of any study procedures. The study included hemodialysis patients older than 18 years of age on a stable hemodialysis regimen for 3 months. Patients were required to be iron replete with baseline TSAT values >20% and ferritin values between <500 ng/ml. Subjects were receiving concomitant recombinant human erythropoetin (Epogen®, Amgen, Thousand Oaks, CA) but were not permitted to receive intravenous or oral

iron for a minimum of 2 weeks prior to the study. Patients who were receiving IV iron prior to the study were receiving iron sucrose at doses ranging from 25 to 100 mg weekly. Patients were excluded for the following reasons: history of allergy or hypersensitivity to any intravenous iron product, hemoglobin value <10 g/dl, recent blood transfusions (within 8 weeks), history of recent bleeding episodes (including increased iron requirements or documented occult gastrointestinal bleeding), active infection, malignancy, history of chronic inflammatory disease (e.g. systemic lupus erythematosus, rheumatoid arthritis), severe hyperparathyroidism (parathyroid hormone concentration >1000 pg/ml), severe malnutrition (serum albumin <2.5 mg/dl) or dialysis inadequacy (urea reduction ratio <65% or Kt/V < 1.2). Controls were healthy, iron replete (TSAT < 35% and ferritin < 100 ng/ml) volunteers with no significant past medical history who were age (±5 years) and sex-matched with enrolled hemodialysis patients.

Study measurements

Intravenous iron administration and blood sample collection

Subjects were admitted to the University of New Mexico General Clinical Research Center on the evening prior to IV iron administration. All patients received standardized, iron restricted (<15 mg) meals. ESRD patients also had restricted phosphorus and potassium. All patients had a 20 gauge 1¾ inch catheter placed on the morning of the study. Catheters were kept patent with normal saline infused at 30–50 ml/h. IV iron products were administered in a random sequence by a computer generated scheme. Intravenous iron products (InFed® (ID), Watson Pharma Inc. and Venofer® (IS), American Regent, Inc. Shirley, NY) were all administered as 100 mg doses of elemental iron by IV push. A test dose of 25 mg is required per the product labeling for ID (Product Information, InFeD®, 2009). Therefore, to maintain temporal consistency, 25 mg test doses were administered for both products. Patients were observed for 5 min after the test dose. The remaining doses were administered over 4 min by IV push to account for the slowest recommended injection rate (IS at 20 mg/min). No subjects were dialyzed during the blood sample collection period to prevent interference of the hemodialysis process itself and potential removal of the analytes being measured. Blood samples were drawn immediately prior to (baseline) and at 60 and 240 min after IV iron administration for measurement of iron indices (NTBI and TSAT), oxidative stress markers (F_2 isoprostane, high sensitivity C-reactive protein (hsCRP), inflammation (IL-1, IL-6, TNF-α and IL-10) and PBMC were isolated for intracellular ROS detection (O_2^-) and mitochondrial membrane potential ($\Delta\psi m$). Serum and plasma were aliquoted and frozen immediately at −80°C for later batch processing. There was a 2 week washout period between each IV iron product administration.

Iron indices

TSAT and NTBI were determined at baseline, 60 and 240 min after each IV iron administration. Serum iron was measured by spectrophotometry using the ascorbate/FerroZine method (CV < 6%). The TSAT was calculated by the following equation: serum iron/TIBC × 100. NTBI was measured using the methods described by Breuer et al. (Breuer and Cabantchik 2001). Briefly, the method involves mobilization of iron with 10 mM oxalate and transfer to fluoresceinated apo-Tf (Fl-Tf). Gallium is present in the assay to prevent the binding of NTBI to unlabelled apo-Tf in the serum sample. NTBI values are derived from the magnitude of quenching of the fluorescence signal of the Fl-Tf. To minimize the possibility of false-positive NTBI values and interference from extraneous factors, an "arbitrary 0" value was determined by analyzing sera from 40 normal controls. The highest value NTBI value from the normal controls was 3.8. Thus, all NTBI values determined in study subjects that were above 3.8 were considered positive. The fluorescence measurements were carried out in 96-well plates with a Tecan GENios plate reader (Zurich, Switzerland) operating at 485/535 nm excitation/emission filter pair (gain = 25). Serum transferrin and ferritin were measured at the University of New Mexico's core laboratory (TriCore Laboratories, Albuquerque, NM) at baseline by standard methodology.

Cytokine activation

Serum was stored at −80°C until analysis. Customized multiplex human cytokine panels were used (Bio-Plex Human Cytokine Singleplex sets, BioRad,

Hercules, CA) for the simultaneous quantification of plasma IL-1β, TNFα, IL-6 and IL-10. All measurements were performed in duplicate. Briefly, the cytokine standards were resuspended in provided assay buffer and then serially diluted according to the manufacturers instructions. A total of 50 µl of reconstituted standard, Quality Controls, or sample (1:4 dilution) was added to each well of a 96-well plate with 50 µl of the bead suspension. The plate was sealed, covered with aluminum foil, and incubated for 30 min at room temperature with agitation on a plate shaker. Then the plate was washed twice with 200 µl/well of wash buffer, removing buffer by vacuum filtration (<100 mmHg) between each wash. This was followed by addition of 25 µl of a multiplex detection antibody stock solution into each well and incubation at room temperature for 30 min. A total of 50 µl of a streptavidin–phycoerythrin solution was added to each well and incubated at room temperature for 30 min. The plate was then analyzed on the Luminex IS100 analyzer (Luminex Inc., Austin, TX, USA). The data were saved and evaluated as median fluorescence intensity using appropriate curve-fitting software (BioPlex Manager 4.1, Hercules, CA). A five parameter logistic method with weighting was used.

Intracellular reactive oxygen species generation and mitochondrial membrane potential

Peripheral blood mononuclear cells were separated from 10 ml of whole blood on Ficoll-Hypaque density gradients, washed twice in PBS (pH 7.4), and centrifuged again at 3,000 rpm and 4°C.

Isolated PBMCs were incubated with Iscoves Modified Dulbecco's Medium for 24 h at 37°C at 5% CO_2. Cell viability was determined by trypan blue exclusion. One aliquot of cells was incubated with 0.5 mM Dihydroethidium (DHE) (Molecular Probes, Eugene, OR) for 15 min and washed three times with chilled Dulbecco's phosphate buffered saline (DPBS) to probe for superoxide (O_2^-) production. A second aliquot of cells were incubated with 1 nM 3,3′-dihexylxacarbocyanine iodide ($DIOC_6(3)$) (Molecular Probes, Eugene, OR) for 15 min and washed three times with DPBS to measure membrane potential collapse ($\Delta\psi m$). Fluorescently labeled cells were analyzed using the BD FACS Scan (Becton–Dickinson, BD Biosciences, San Jose, CA) and CellQuest Pro Version 5.2 (Rockville, MD).

C-reactive protein

Serum was isolated from whole blood collected in 7 ml BD vacutainer tubes with SST II preservative. High sensitivity C-reactive protein (hsCRP) was determined by chemiluminescence (Immulite, Diagnostic Products Corporation, Los Angeles, CA).

Plasma F_2 isoprostane

Five ml of whole blood was collected into a BD vacutainer containing 5.4 mg of K2 EDTA. Plasma F_2 isoprostanes were analyzed by GC/negative-ion chemical ionization and electron ionization MS as previously described (Morrow et al. 1990). Sample analysis was performed at the Human Analytical Isoprostane Core Facility at Vanderbilt University, Nashville, TN.

Statistical analysis

The primary endpoint for the study was the appearance of NTBI. Data are reported as mean ± SEM or mean ± SD where indicated. Repeated measures ANOVA with grouping factors and time as a repeated measure, followed by Fisher's protected least significant difference test, was used to assess differences in NTBI concentrations at baseline ($t = 0$) 60 and 240 min after iron administration. If data were normally distributed, a two-tailed Kruskal–Wallis test was used to assess differences between groups. Wilcoxon's signed rank test will be used to analyze paired data. All data were analyzed using SPSS v11 (Chicago, IL). A P value <0.05 is considered significant.

Based on our preliminary data and available literature (Pai et al. 2007; Lim et al. 1999), a sample size of 10 ESRD patients will be adequate to detect a 7 pg/ml in IL-6 secretion with a power of 80% and an α of 0.05. Concerning the other parameters, our sample size is adequate to detect an effect size of 1.2 with 80% power.

Results

Ten ESRD patients and four healthy controls were enrolled in the study. Demographic characteristics of the study patients are listed in Table 1.

Table 1 Baseline demographic and laboratory parameters

Parameter	ESRD patients ($n = 10$)	Healthy controls ($n = 4$)
Age (years)	57.7 (3)	43.5 (7.9)
Sex (%)	Male (50%)	Male (50%)
	Female (50%)	Female (50%)
Ethnicity (%)	Hispanic (40%)	White (50%)
	White (30%)	Black (50%)
	Native American (20%)	
	Black (10%)	
Transferrin saturation (mg/dl)	28.1 (3.4)	32 (11.4)
Ferritin (ng/ml)	423 (67)	65 (37.9)
C-reactive protein (mg/l)	13.4 (3.2)	3.1 (1.5)*
Time on hemodialysis (months)	43.5 (1.37)	N/A
Hemodialysis adequacy Kt/V	1.4 (0.1)	

Data or reported as mean (SD), *N/A* not applicable, * $P < 0.05$ ESRD vs. healthy controls

NTBI in hemodialysis patients and healthy controls

Patients with ESRD had higher NTBI concentrations compared to healthy controls after IV iron administration (Fig. 1a, b). ESRD patients also had greater transferrin oversaturation and NTBI appearance following ID administration versus healthy controls. Only one healthy control patient had detectable NTBI after ID, measured at 240 min post infusion (0.76 μM). The NTBI area under the concentration–time curve was significantly higher in ESRD patients who received IS compared to ID, indicating increased exposure to labile iron (Table 2).

Serum cytokine activation

At baseline, all measured cytokine concentrations were significantly elevated in ESRD patients compared to healthy controls (Fig. 2a–c). The most pronounced effect of both IV iron products was observed with IL-6 concentrations in ESRD patients ($P < 0.05$ for both IS and ID vs. baseline) whereas levels of IL-6 were relatively undetectable in healthy controls at baseline and after IV iron (Fig. 2a). Serum concentrations of TNF-α increased significantly with both ID and IS administration in both ESRD patients and healthy controls (Fig. 2b). The TNF-α Cmax after ID was significantly higher in ESRD patients than healthy controls. IL-1 concentrations increased after both products in both ESRD and healthy controls (Fig. 4, $P < 0.05$). The anti-inflammatory cytokine IL-10 (Fig. 2c) did not change significantly

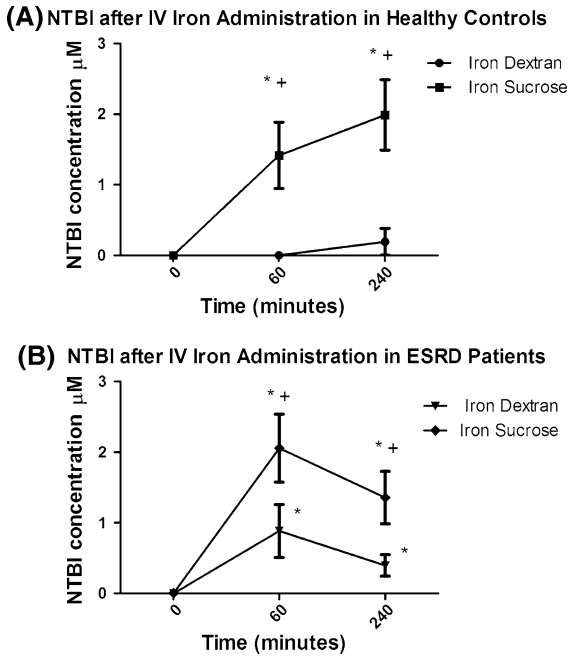

Fig. 1 NTBI after IV iron sucrose and iron dextran administration in healthy controls (**a**) and ESRD patients (**b**)

with IV iron administration. Post-infusion cytokine concentrations did not differ between IS and ID administration in either study group.

Intracellular reactive oxygen species generation and mitochondrial membrane potential

Only one healthy control had an increase in intracellular ROS generation measured by DHE fluorescence, measured at 60 and 240 min after IV ID administration

Table 2 Area under the serum concentration–time curve (AUC) after IV iron sucrose and iron dextran

	AUC_{0-240} (μM*min/l)*
ESRD	
ID	74 ± 23
IS	202 ± 52**
Healthy controls	
ID	92.2 ($n = 1$)
IS	281 ± 59

ID iron dextran, *IS* iron sucrose, *ESRD* end-stage renal disease

* Data reported as mean ± SEM, ** $P < 0.05$ ID vs. IS

(20 and 2%, respectively). Among the ESRD patients treated with ID, 83% had an increase in intracellular ROS detected at 60 min and 50% had increased ROS at 240 min (Fig. 3). Administration of IS resulted in increases in intracellular ROS in 50% of patients at 60 min and 75% of patients at 240 min (Fig. 3). This suggests there may be different time courses for ROS generation among the IV iron products.

All of the healthy controls had a loss of mitochondrial membrane potential after IS and 50% had loss of ψm with ID (Fig. 4). In ESRD patients, more patients (80%) had loss of ψm after ID, with most patients having a nadir at 240 min. Similarly, after IS 42% of patients had reduced ψm (42%) at 240 min after administration.

Inflammation and lipid peroxidation

Baseline hsCRP was significantly higher in ESRD patients compared to healthy controls, consistent with chronic inflammation that is well documented in the ESRD population (Table 1). Similarly, F_2 isoprostane, a sensitive direct marker of in vivo free radical oxidative damage to membrane phospholipids, was elevated at baseline in ESRD patients compared to healthy controls (0.159 ± 0.022 vs. 0.065 ± 0.004 ng/ml, $P < 0.05$). There were no significant differences in F_2 isoprostane concentrations after IV iron administration in ESRD or healthy controls.

Discussion

Intravenous iron therapy has been shown to improve hemoglobin response to ESA therapy in hemodialysis patients, thus firmly establishing it's place in the treatment of anemia of CKD (KDOQI 2006). Intravenous iron use is likely to significantly increase under the new Centers for Medicaid and Medicare Services Conditions for Coverage that will mandate "bundle" reimbursement for dialysis services and intravenous drugs (ESRD Conditions Final Rule, April 15, 2008). Given that ESAs are far more expensive than IV iron and have come under scrutiny because of several studies (Besarab et al. 1998; Singh et al. 2006; Drüeke et al. 2006; Pfeffer et al. 2009) associating their use to achieve hemoglobin targets >13 g/dl with increased mortality, it is likely that IV iron will be used more aggressively to achieve current hemoglobin goals. Despite the well established efficacy of IV iron, safety concerns remain regarding the systemic effects of the pro-oxidant nature of these products inducing oxidative stress that has been associated with inflammation and cardiovascular disease (Zager 2006). The effect of IV iron on adverse events and mortality among ESRD patients has been difficult to elucidate from epidemiologic analysis of large hemodialysis databases because IV iron is typically always used concomitantly with ESA therapy (Feldman et al. 2004; Kalantar-Zadeh et al. 2005). Thus, translational studies have sought to determine not only the specific pro-oxidant effects but also whether differences exist between these compounds that vary widely with regard to carbohydrate shield chemistry, pharmacokinetic profile and generation of NTBI (Zager et al. 2002; Pai et al. 2007; Kuo et al. 2008).

Studies of IV iron-induced oxidative stress have relied principally on the hypothesis that NTBI (e.g. free ferric iron) is available to engage in the Fenton–Haber-Weiss reaction whereby Fe^{3+} is reduced then reacts with hydrogen peroxide to ultimately generate the highly reactive hydroxyl radical, that can rapidly incite deleterious redox reactions with molecules in close proximity such as plasma proteins (e.g. low density lipoprotein) or DNA (Adibhatla and Hatcher 2010). In this study, we found significantly higher generation of free iron after bolus infusion of the small molecular weight iron (IS) versus a large molecular weight compound (ID) using a fluorescence based assay for NTBI (Breuer and Cabantchik 2001). The NTBI appearance observed in this study is likely due to both oversaturation of transferrin binding sites after reticuloendothelial processing of

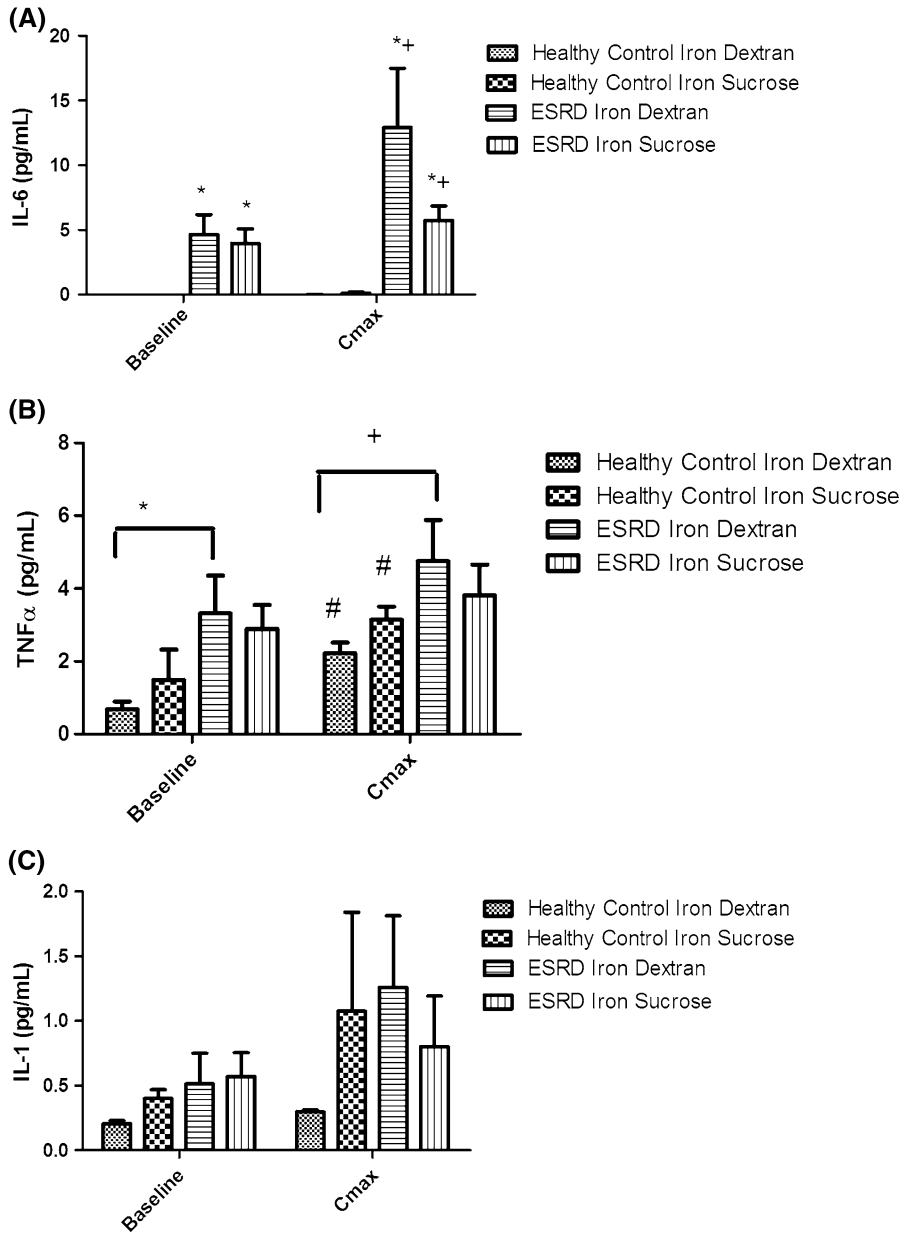

Fig. 2 Serum cytokine concentrations after IV iron. **a** TNF-α concentration, * $P < 0.05$ HC vs. ESRD ID baseline, + $P < 0.05$ HC vs. ESRD ID Cmax, # $P < 0.05$ BL vs. Cmax HC (ID and IS), $P =$ NS BL vs. Cmax for ESRD (ID and IS). **b** Serum IL-6, * $P < 0.05$ HC vs. ESRD all comparisons, + $P = <0.05$ BL vs. Cmax for ESRD (ID and IS). **c** Serum IL-1, $P =$ NS all comparisons

the iron carbohydrate complex and direct donation of iron from the iron formulations to transferrin (Balakrishnan et al. 2009; Van Wyck et al. 2004). The NTBI assay is not able to distinguish from which of the aforementioned pathways the iron is derived from and this is a limitation of this measure for both clinical and research use. The optimal way to resolve this issue would be to formulate ^{59}Fe-labeled IV iron complexes and examine the pharmacokinetic profile to understand the contribution of endogenous serum iron versus exogenous formulation derived iron. Such a formulation has only been created for iron dextran and was only studied in the late 1960s on the context of incorporation into hemoglobin and not with regard to direct donation to transferrin (Henderson and Hillman 1969). The most commonly used clinical laboratory measure of "available iron", transferrin saturation (serum iron/total iron binding capacity × 100), cannot quantify the amount of NTBI that may appear after IV iron formulation. Oversaturation

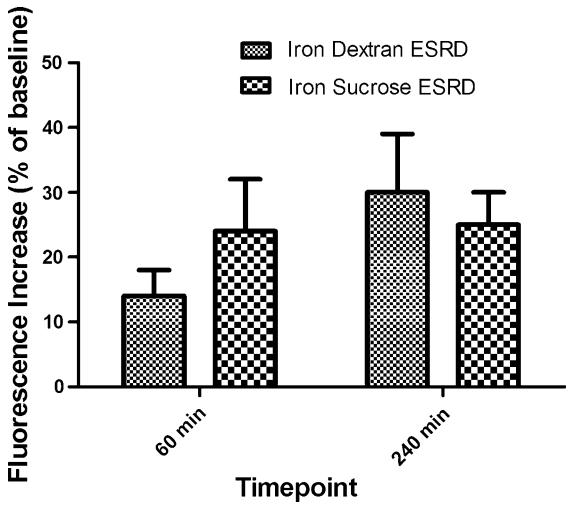

Fig. 3 Intracellular ROS generation after IV Iron in ESRD patients

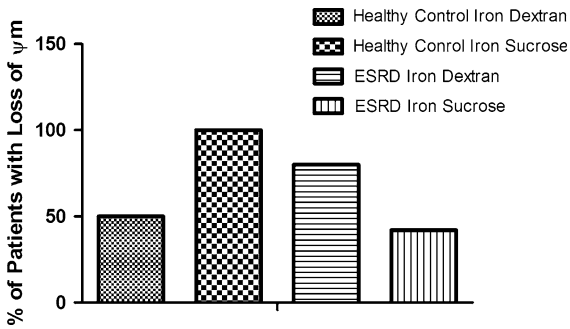

Fig. 4 Mitochondrial membrane potential loss after IV iron

of transferrin with this assay would be reported as ">100%". This indicates likely appearance of NTBI, but does not provide the clinician with any information with which to further guide iron therapy. However, it should be noted that NTBI can be present without the transferrin saturation being reported as >100% and likely indicates iron that is loosely bound to other plasma proteins. In a previous study, (Pai et al. 2007) we showed a significant positive correlation between NTBI and transferrin saturation ($r^2 = 0.45$, $P < 0.001$) after iron sucrose administration.

Despite large differences in NTBI appearance that corresponded to increased exposure to NTBI after IS, cytokine activation, intracellular ROS generation and mitochondrial membrane potential collapse was essentially the same between the two products. This suggests a number of other potential relationships may exist that require further investigation. Firstly, it is possible that the acute effect of NTBI on intracellular ROS generation and possible subsequent activation of NFκB and cytokine transcription exhibits a sigmoidal dose response curve that is rate limited by cellular uptake of NTBI (Shvartsman et al. 2007). That is, only concentrations of NTBI up to an unknown maximum or ceiling concentration can be transported intracellularly and induce intracellular ROS generation and ultimate cytokine activation. Beyond which there is no further deleterious effect on the cell and the remaining NTBI is subsequently "quenched" by transferrin as binding sites become available and/or reacts with plasma proteins. Few of the translational studies have examined the temporal relationship between NTBI generation and oxidative stress. In studies of healthy rats given five doses of five different IV iron products, transferrin saturation increased however the values were not greater than 100% making transferrin saturation unlikely (Toblli et al. 2010). No assessment of NTBI was done so the appearance of NTBI cannot be excluded, however, the serum was sampled 1 week after the dose of IV iron. The NTBI exposure is very transient in a healthy animal model, and thus it is likely that the NTBI generated engaged in immediate reactions and was gone by the time the serum was obtained. Secondly, it is possible that the NTBI generation is only one part if the IV iron-induced oxidative stress puzzle. Tobilli et al. showed that the small molecular weight IV iron sodium ferric gluconate and two iron dextran products (INFed® and Dexferrum®), exhibit the most prominent staining for iron deposition and IL-6 in major organs as well as the highest elevation in lipid peroxidation (TBARS) and antioxidant enzymes (catalase and Zn superoxide dismutase) compared to iron sucrose and ferric carboxymaltose (a large molecular weight compound) (Toblli et al. 2010). This disparate toxicity profile may suggest a contribution of the carbohydrate shield to either direct toxicity or to transport into cells. The authors suggest the enhanced toxicity may be an effect of non-metabolized iron which may be plausible for the high molecular weight compounds that have slow clearance rates (Pai et al. 2010; Henderson and Hillman 1969). However, pharmacokinetic studies of sodium ferric gluconate have shown rapid incorporation of the drug into the body iron pool, limiting the likelihood of prolonged concentrations of unmetabolized iron (Seligman et al. 2004). Thus, the

contribution of the carbohydrate portion of the complex warrants further investigation.

Our study documented increased intracellular ROS in ESRD patients but not healthy controls. In ESRD patients, antioxidant systems are depressed, thus there is less ability to neutralize intracellular ROS. ROS, including superoxide, are known modulators of signal transduction. Cytokine activation observed in this study may be, in part, secondary to ROS-mediated NFκB or other pathway(s) activation. Mitochondrial membrane collapse occurred more frequently in healthy controls, a paradoxical finding given that ROS are the principal cause of impaired mitochondrial respiratory drive and subsequent membrane potential loss. However, one hypothesis purports that the chronic pro-inflammatory stimuli that inundates ESRD patients causes profound inhibition of oxidative phosphorylation that may result in a compensatory hypertrophy process leading to a vicious cycle of continuous ROS production that cannot be sufficiently neutralized by intracellular antioxidant systems (Granata et al. 2009). Thus, the lower loss of membrane potential we observed in ESRD patients may be due to a compensatory mitochondrial dysregulation in hemodialysis patients and should be studied further.

Pro-inflammatory cytokines were significantly increased after administration of both IV iron products, however, the IL-6 response was most profound and occurred only in ESRD patients. IL-6 has been robustly linked to cardiovascular disease and has a superior predictive value for mortality in ESRD patients on hemodialysis (Panichi et al. 2004). IL-6 is also the predominant inflammatory mediator of hepcidin expression, a 25-amino acid peptide that is a pivotal modulator of tissue iron stores and serum iron availability (Fleming 2008). In this study both agents induced significant IL-6 production. Reticuloendothlial (RES) blockade and dysfunctional iron metabolism is common in hemodialysis patients. RES blockade appears to be mediated, at least in part, by elevated hepcidin that endocytoses ferroportin channels and impairs iron bioavailability (Zaritsky et al. 2009). Thus, there could be a direct relationship between IV iron administration and induction of hepcidin which points to a possible "vicious cycle" phenomenon where more IV iron administration could contribute to RES blockade and suboptimal hemoglobin responses which leads clinically to more iron administration. Whether this may be more related to a specific IV iron compound or could be ameliorated by reduced doses or prolonged infusions warrants further exploration in clinical trials.

Finally we examined CRP and F_2 isoprostane biomarkers of inflammation and lipid peroxidation, respectively. CRP was significantly elevated compared to healthy controls which is well-characterized in ESRD patients (den Elzen et al. 2006) Accordingly, F_2 isoprostane was elevated in ESRD patients compared to controls, however, this biomarker did not respond to acute IV iron administration. Collectively, these markers convey the marked inflammatory burden in ESRD, however they may not be sensitive enough to detect acute inflammatory changes, perhaps because of the biochemically complex inflammatory milieu.

Our results need to be considered in the context of several limitations of our study. There were a small number of patients involved in the study, thus requiring confirmation of our results in a larger clinical trial. We studied the IV iron formulations in ESRD patients on non-dialysis days in a controlled clinical research unit setting. This limits the generalizability of these findings as IV iron is always administered during the hemodialysis session. However, our aim in conducting the study with this design was to attempt to distinguish oxidative stress induction by IV iron versus the hemodialysis process it self which has been well documented in other studies (Raj et al. 2007). It is also important to note that IV iron formulations are not significantly removed by the hi-flux hemodialysis process (Manley and Grabe 2004). Additionally, we only used two of the four available IV iron compounds and therefore our results cannot necessarily be extrapolated to other products.

In summary, this translational study demonstrates that despite differential NTBI exposure profiles, iron dextran and iron sucrose induced similar intracellular ROS generation and IL-6 activation in ESRD. The marked differences in ESRD patients in baseline cytokine concentrations and intracellular responses to IV iron administration should be studied further to determine implications for pivotal signaling pathways.

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