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Effect of intravenous vitamin C on cytokine activation and oxidative stress in end-stage renal disease patients receiving intravenous iron sucrose

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Abstract Reticuloendothelial blockade in hemodialysis patients prevents optimal intravenous (IV) iron utilization. Vitamin C has emerged as a potential therapy to improve anemia treatment by enhancing iron mobilization. However, Vitamin C can act as a pro-oxidant in the presence of iron. This was a prospective, open-label, crossover study. Thirteen patients with end-stage renal disease on hemodialysis and four healthy controls were assigned to receive 100 mg of IV iron sucrose (IS) or 100 mg of IV IS co-administered with 300 mg of IV Vitamin C (IS + C) in random sequence. Serum samples for IL-1, IL-6, TNF-α and IL-10 and non-transferrin bound iron were obtained at baseline, 45 min and 105 min post study medication administration. Peripheral blood mononuclear cells 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 F2-isoprosatane concentration. Both IS and IS + C were associated with increased plasma F2-isoprostanes concentrations post-infusion. Maximal plasma F2-isoprostane concentrations after IS + C were significantly elevated from baseline (234 ± 0.04 vs. 0.198 ± 0.028 ng/mL, $p = 0.02$). After IS + C, IL-1, IL-6, IL-10, and TNF-alpha were significantly elevated compared to baseline. After IS alone only IL-6 was noted to be elevated. Intracellular production of H_2O_2 and loss of mitochondrial membrane potential ($\Delta\psi$m) was observed after IS while IS + C was associated with increased $O_2^{\cdot-}$ production. Both IS and IS + C induced serum cytokine activation accompanied by lipid peroxidation, however, IS + C induced higher plasma concentrations of F2-isoprostanes, IL-1, IL-10, and TNF-α post-infusion. Long-term safety studies of IV iron co-administered with Vitamin C are warranted.

Keywords Oxygen radicals · Inflammation · Cytokines · Iron sucrose · Vitamin C · Hemodialysis

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Introduction

Intravenous (IV) iron supplementation is administered to optimize red blood cell production by erythropoiesis stimulating agents to reach target hemoglobin

concentrations in end-stage renal disease patients (ESRD) on hemodialysis (HD) (KDOQI 2007). An estimated 90 % of HD patients have hemoglobin fluctuation patterns (Gilbertson et al. 2008). This hemoglobin variability has been attributed to erythropoiesis-stimulating agents (ESA)-resistance characterized by not reaching target hemoglobin goals despite high doses of ESAs (e.g. >400,000 units of epoetin alfa/month). ESA-resistance is also characterized by reticuloendothelial system (RES) blockade impairing iron mobilization. Clinically, RES blockade manifests as high serum ferritin usually coincident with low transferrin saturation (TSAT) concentrations, indicating impaired mobilization and transfer of iron. Other causes of ESA-resistance include chronic oxidative stress and inflammation and more recently, hepcidin up-regulation (de Francisco et al. 2009). Recent studies to improve anemia management in ESRD patients have focused on adjuvant therapies, such as Vitamin C to circumvent impaired iron delivery from RES blockade (Attallah et al. 2006; Chan et al. 2005; Sirover et al. 2008). The use of Vitamin C as an adjuvant is based on its anti-oxidant properties and its ability to increase iron bioavailability by mobilizing iron from the RES to transferrin and hastening release of iron from ferritin (Berger et al. 1997; Sturm et al. 2005). Short-term Vitamin C supplementation studies have shown improvement in anemia parameters in patients with laboratory profiles consistent with inflammation and RES blockade (Keven et al. 2003; Sturm et al. 2005; Tarng et al. 2004a, b).

Despite favorable effects on hemoglobin, safety concerns remain with IV iron and Vitamin C. In the presence of a transition metal such as iron, Vitamin C is a strong reducing agent with the ability to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). Fe^{2+} drives the production of reactive oxygen species (ROS), most notably the highly reactive hydroxyl radical ($^{\cdot}OH$) and the superoxide radical ($O_2^{\cdot-}$) (Weiss and Gordeuk 2005, Buettner and Jurkiewicz 1996). ROS may cause protein, lipid, and oxidative damage of DNA potentially accelerating endothelial damage, atherosclerosis, and cardiomyopathy in ESRD patients (Locatelli et al. 2003; Drüeke et al. 2002; Rooyakkers et al. 2002). Under normal homeostatic conditions this reaction is tightly regulated and unlikely to cause deleterious effects. However, this interaction has not been fully explored in patients receiving pharmacologic doses of IV iron and Vitamin C concomitantly.

Few studies have specifically examined the pro-oxidant effects of Vitamin C when given with IV iron supplementation in ESRD patients (Eiselt et al. 2006; Shi et al. 2005). Eiselt et al. (2006) observed an increase in lipid peroxidation after 100 mg of IV iron sucrose (IS) was co-administered with a 2 mg/minute infusion of Vitamin C during one HD session. In contrast, Shi et al. (2005) reported attenuation of lipid peroxidation when Vitamin C was co-administered with IV iron over a single HD session. Other studies have explored the antioxidant and pro-oxidant properties of Vitamin C in ESRD patients irrespective of iron usage with equivocal findings (De Vriese et al. 2008; Tarng et al. 2004a, b).

The principal limitations of these previous studies include; use of biomarkers providing a limited evaluation of inflammation and oxidative stress, exclusion of patients with iron overload or who are receiving IV iron, and the potential confounding effect of the HD process itself on concentrations of bio-markers (e.g. removal of thiobarbituric acid reactive substances (TBARS)). The purpose of this pilot study was to evaluate the acute oxidative and pro-inflammatory effects of Vitamin C supplementation in ESRD patients with presumed RES blockade receiving IV iron therapy using a systems biology approach.

Methods and materials

This was a prospective, randomized, open-label, crossover study of the effects of IV Vitamin C supplementation on intracellular ROS production, lipid peroxidation, oxidative stress and cytokine activation in ESRD patients on HD receiving IV iron therapy. The study was approved by the University of New Mexico Human Research Review Committee. All patients provided informed consent.

Patient selection

Inclusion criteria were; patients greater than 18 years, on stable HD regimen for 90 days or more, receiving concomitant recombinant human erythropoietin (Epogen ®, Amgen, Thousand Oaks, CA), had not received IS or Vitamin C supplements within the past 2 weeks, ferritin >500 ng/L and TSAT of <30 %.

Exclusion criteria included history of allergy or hypersensitivity to any IV iron product or IV Vitamin C, recent transfusions (within 8 weeks), history of

recent bleeding episodes, active infection or malignancy, history of chronic autoimmune inflammatory disease, severe hyperparathyroidism (iPTH > 1000 pg/mL), severe malnutrition (serum albumin <2.5 mg/dL), HD inadequacy (URR <65 % or Kt/V <1.2), anti-inflammatory drugs within last 3 months (i.e. corticosteroids, immunosuppressants), and women of child-bearing potential who did not agree to use highly effective birth control measures. A group of iron replete (TSAT <35 % and ferritin <100 ng/mL) volunteers with no significant past medical history served as healthy controls (HC).

Study design

Eligible ESRD patients and HC were randomly assigned to receive IS (Venofer® (IS), American Regent, Inc.) alone or IS + C in a two way-crossover design. Study procedures took place on a non-HD day for ESRD patients. In the IS arm, IS 100 mg was given by IV push over 5 min. In the IS + C arm, a 15 min infusion of IV Vitamin C 300 mg was administered at the end of the 5 min IV IS push. Vital signs were measured every 15 min for 1 h post-administration of the study medication and at each blood draw time point. Blood samples were drawn at baseline, 45 min, 105 min, and 24 h after study medication administration for measurement of iron, oxidative, and inflammatory markers described below. There was a 2 week washout period between each treatment.

Measurement of non-transferrin bound iron levels

Non-transferrin bound iron (NTBI) was measured using the methods described by Breuer and Cabantchik (2001). To minimize the possibility of false-positive NTBI values and interference from extraneous factors, an "arbitrary 0″ value was set at 1.6 determined by taking the highest NTBI value by analyzing sera from 10 controls. The fluorescence measurements were carried out in 96-well plates with a Tecan GENios™ plate reader operating at 485/535 nm excitation/emission filter pair (gain = 25).

Measurement of F2-isoprostanes

F2-isoprostanes, specifically 8-iso-PGF2α, were analyzed by gas chromatography/negative-ion chemical ionization and electron ionization mass spectrometric (Milne et al. 2007). Sample Analysis was performed at the Human Analytical Isoprostane Core Facility at Vanderbilt University, Nashville, TN supported by NIH Grant DK20593.

Analysis of serum cytokine levels

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, TNF-α, IL-6 and IL-10. Analysis was performed on the Luminex IS100™ analyzer (Luminex Inc.). The data were saved and evaluated as median fluorescence intensity using appropriate curve-fitting software (BioPlex Manager 4.1).

Flow cytometric analysis of intracellular ROS production

Isolated peripheral blood mononuclear cells (PBMC) 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 dihydrorhodamine 123 (DHR-123) (Molecular Probes) for 45 min to probe for hydrogen peroxide (H_2O_2) production. These cells were then incubated with 0.5 mM dihydroethidium (DHE) (Molecular Probes) for 15 min to probe for $O_2^{\cdot-}$ production. A second aliquot of cells were incubated with 1 nM 3,3′ -dihexylxacarbocyanine iodide ($DIOC_6(3)$) (Molecular Probes) for 15 min to measure membrane potential ($\Delta\psi m$). Fluorescently labeled cells were analyzed using the BD FACS™ Scan (Becton–Dickinson, BD Biosciences) and CellQuest Pro™ Version 5.2.

Vitamin C analysis

Approximately 5 mL of whole blood was collected into a BD vacutainer tube containing 5.4 mg of K2 EDTA. This sample was then centrifuged at 3000 rpm and 2 mL of undiluted plasma was aliquoted into a 15 mL cryogenic tube containing 40 mg oxalic acid preservative and analyzed at ARUP Laboratories.

C-reactive protein (CRP) analysis

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 using chemiluminescent reactions (Immulite®, Diagnostic Products Corporation).

Data analysis

Data are reported as mean ± standard error of the mean (SEM). Repeated measures ANOVA with grouping factors and time as a repeated measure, followed by post hoc *t*-tests, were used to assess differences between groups at baseline, 45 min, 105 min, and maximal plasma concentration (Cmax) after study medication administration. Categorical data were analyzed by Pearson's χ^2 test. All data analysis was performed on SPSS v 14.0.

Results

A total of 13 ESRD patients and 4 HC were enrolled in the study. The baseline demographics and laboratory parameters of the ESRD patients and HC are shown in Table 1. ESRD patients had presumed RES blockade (ferritin, 703 ± 102 ng/mL; TSAT, 21 ± 2 %) with high weekly epoetin alfa requirements (285 ± 90 IU/kg/week) and elevated hsCRP (7.3 ± 4.9 mg/L), indicative of ESA resistance and underlying inflammation.

Appearance of NTBI after IS or IS + C

There were no significant differences in NTBI appearance after study medication administration between the ESRD groups and the HC (Fig. 1a, b). However, both the 45 and 105 min time points were elevated from baseline within the ESRD and HC groups ($p < 0.01$).

Lipid peroxidation and inflammation

F2-isoprostane, a sensitive direct marker of in vivo free radical oxidative damage to membrane phospholipids, was elevated at baseline and post study medication administration in both ESRD groups when compared to HC (Fig. 2). F2-isoprostane increased from baseline in both ESRD groups, but was only significantly increased in the IS + C group (0.198 ± 0.028 vs. 234 ± 0.04 ng/mL, $p = 0.022$) (Fig. 2).

Cytokine activation

At baseline and post-infusion, all measured cytokine concentrations were elevated in ESRD patients compared to HC. IL-1 concentrations were increased after both IS and IS + C administration in ESRD patients, however this was only significant after IS + C (0.81 ± 0.12 vs. 1.95 ± 0.53 pg/mL, $p = 0.038$, Fig. 3a). IL-6

Table 1 Patient Demographics

Parameter	ESRD patients ($n = 13$)	Healthy controls ($n = 4$)
Age (years)	57.6 ± 2.8	43.4 ± 7.9
Sex (Male; *n* [%])	7 (53.8)	2 (50)
Etiology of ESRD (*n*[%])		
Diabetes mellitus	6 (46.2)	–
Hypertension	5 (38.5)	–
Other	2 (15.3)	–
HD Vintage (months)	74.4 ± 24.9	–
Kt/V	1.62 ± 0.23	–
Erythropoietin (IU/kg/week)	285 ± 90	–
Hemoglobin (g/dL)	12.19 ± 0.38	–
TSAT (%)	20.8 ± 2.02	32 ± 11.4*
Ferritin (ng/mL)	703 ± 102	65 ± 37.9*
hsCRP (mg/L)	7.3 ± 4.8	2.6 ± 1.6*
Albumins (g/dl)	3.79 (0.09)	–
Total Cholesterol (mg/dL)	180 ± 18.8	–
PTH(pg/ml)	404 ± 64	–

Data reported as mean ± SE unless otherwise indicated

ESRD end stage renal disease, *HD* hemodialysis, *Kt/V* dialysis adequacy, *TSAT* transferrin saturation, *hsCRP* high-sensitivity C-reactive protein, *PTH* parathyroid hormone

*$p < 0.05$ ESRD vs. healthy control

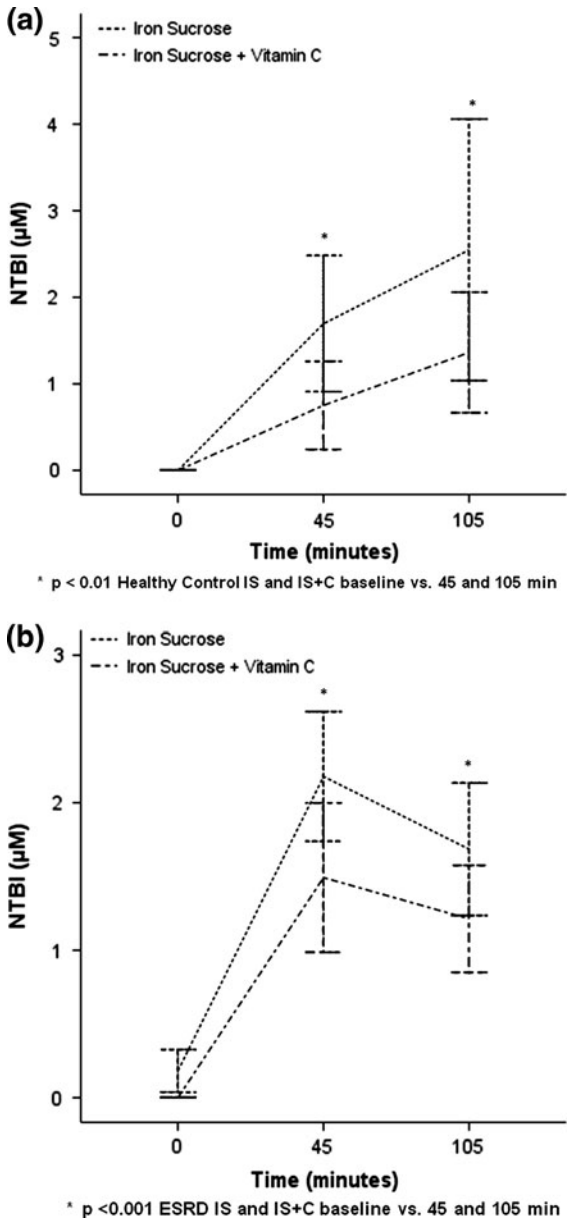

Fig. 1 Non-transferrin bound iron after study medication administration in healthy controls (**a**) and ESRD patients (**b**)

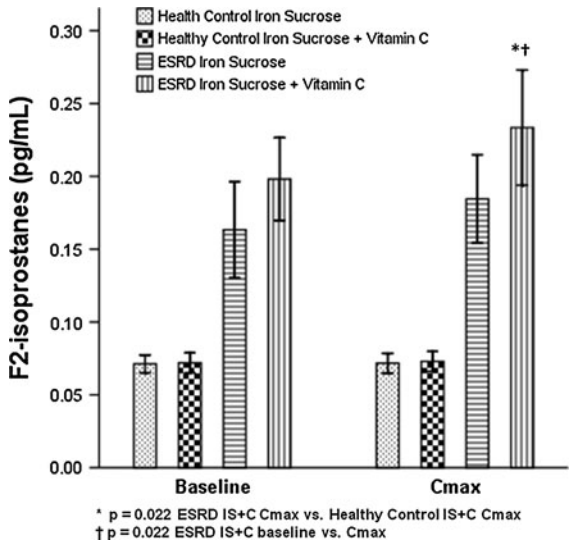

Fig. 2 Lipid peroxidation before and after study medication administration

concentrations increased in ESRD patients post IS and IS + C (Fig. 3b). IL-10 concentrations were significantly increased after IS + C in ESRD patients while HC exhibited no change (Fig. 3c). TNF-α, a strong predictor of mortality among patients on HD, increased significantly after IS + C in ESRD patients (Fig. 3d).

Intracellular reactive oxygen species generation and mitochondrial membrane potential ($\Delta\psi$m)

Intracellular O_2^- generation increased in only 1 HC from baseline after IS and IS + C. In ESRD patients, 63 % and 71 % of the patients in the IS and IS + C groups had an increase from baseline in DHE fluorescence (mean increase 10.5 ± 5.9 vs. 25.7 ± 11, respectively, $p = 0.417$, Fig. 4). H_2O_2 generation increased in all HCs after IS and IS + C (mean increase 153.9 ± 72.2 vs. 117.4 ± 46.1, $p = 0.713$, Fig. 4). In ESRD patients, 88 % and 71 % of the patients in the IS and IS + C groups had an increase from baseline in DHE fluorescence (mean increase 65.4 ± 25.3 vs. 42.7 ± 14.8, $p = 0.504$, Fig. 4).

HCs had a 75 % and 33 % loss of mitochondrial membrane ($\Delta\psi$m) post IS and IS + C, respectively ($p = 0.486$). In ESRD patients, 100 % of patients had loss of membrane potential ($\Delta\psi$m) after IS compared to 57 % after IS + C ($p = 0.077$).

Discussion

This is the first study to our knowledge to evaluate acute inflammatory and oxidative stress biomarkers in vivo after co-administration of IV iron with Vitamin C in ESRD patients using a systems biology approach. Adjuvant therapies to improve response to anemia

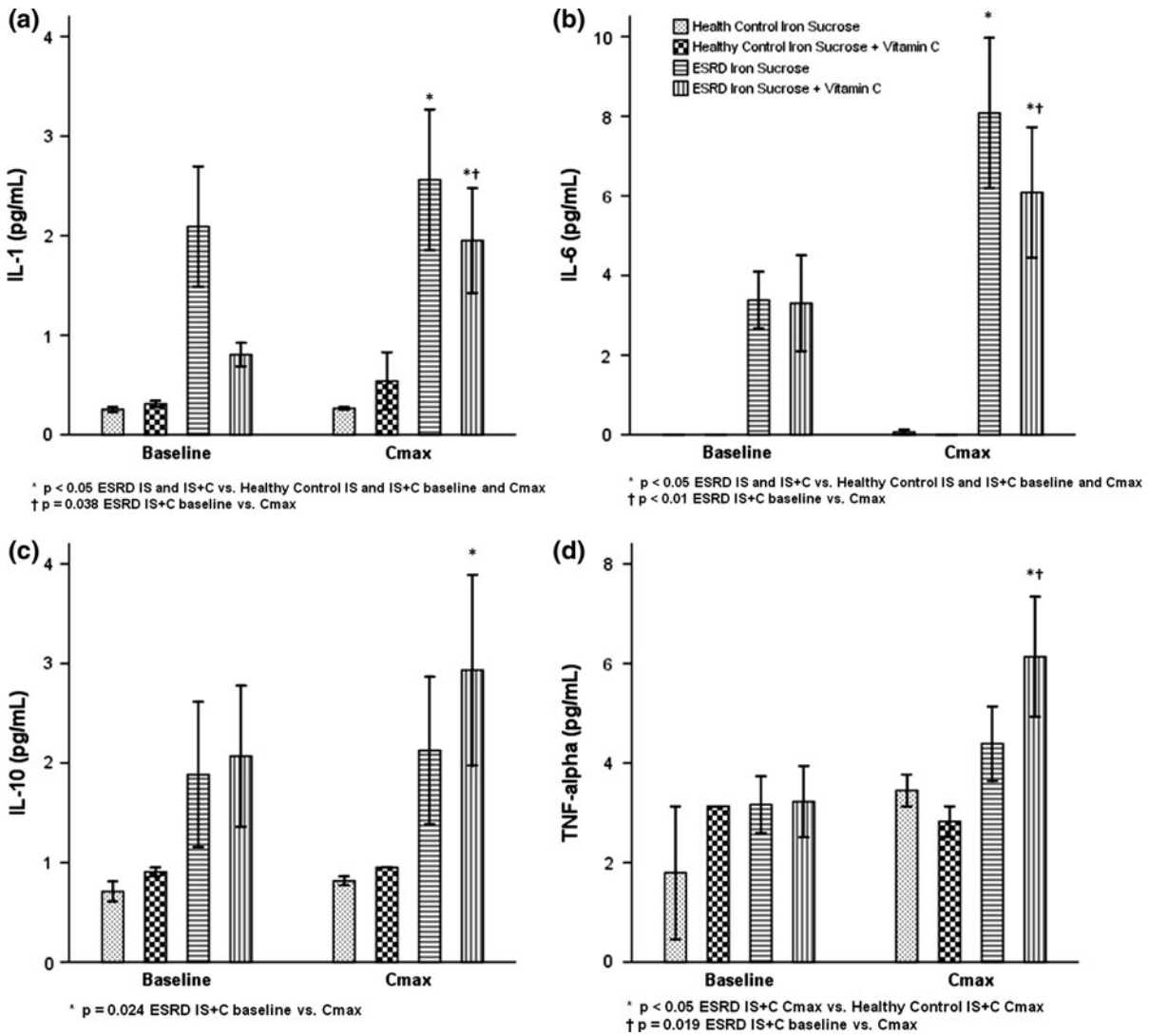

Fig. 3 Serum cytokine concentrations before and after study medication administration. **a** Serum IL-1. **b** Serum IL-6. **c** Serum IL-10. **d** Serum TNF-α

treatment are increasingly being considered by clinicians in the USA, especially in light of the new bundled payment structure initiated January 1, 2011 by the Centers for Medicaid and Medicare Services (2008). The new bundled payment reimburses HD units as a composite rate comprised of dialysis treatment, laboratory services and drugs whereas the latter two items were formally separately billable. The implications of the bundle on ESA and iron therapy have not been fully elucidated. Data from Japan's imposed bundled reimbursement structure in 2006 clearly show more aggressive IV iron use principally to reduce use of the more expensive ESAs (Hasegawa et al. 2010). Thus, it remains critically important to understand the toxicity profile of IV iron especially when administered concomitantly with inexpensive, widely used adjuvants such as Vitamin C in efforts to optimize anemia treatment at minimal cost.

As previously described, the production of the $^{\cdot}OH$ is driven by the oxidation of Fe^{2+} to Fe^{3+}. This reaction is potentiated in the presence of Vitamin C by the redox cycling of Fe^{3+} (e.g. from ferric iron-carbohydrate complexes) to Fe^{2+} generating supra-physiological production of $^{\cdot}OH$, leading to potential oxidative damage of lipids, proteins, and DNA as well as inducing nuclear factor κB (NFκB) activation and

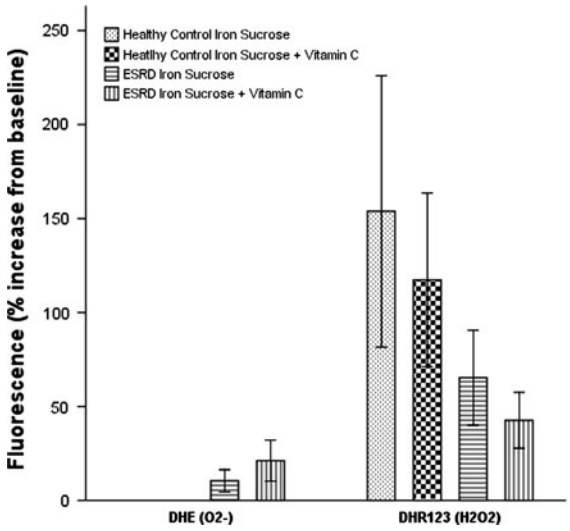

Fig. 4 Intracellular reactive oxygen species generation after study medication administration

pro-inflammatory cytokine transcription (Buettner and Jurkiewicz 1996; Shi et al. 1999). The interaction between iron and Vitamin C in vivo is complex and most studies have shown limited toxicity (Buettner and Jurkiewicz 1996). However, the majority of studies have been conducted in healthy patients with intact intra- and extracellular antioxidant systems and preserved iron metabolism. In contrast, HD patients are well known to have limited antioxidant defense systems and dysfunctional intra- and extracellular iron metabolism due to chronic inflammation (Sonnweber et al. 2011; Otaki et al. 2004).

Studies conducted in vitro have shown NTBI available for direct donation to transferrin from iron-polysaccharide formulations (Esposito et al. 2002). The concentration of NTBI has been shown to be increased in the presence of Vitamin C (Stefansson et al. 2008). This is the first in vivo study to investigate co-administration of IV Vitamin C with clinically relevant doses of IV IS. While administration of both IS and IS + C increased NTBI, there was no difference between the treatments. These data indicate lower NTBI appearance after IS + C. This may represent a rapid reduction of Fe^{3+} released from IS to Fe^{2+} which is not detected by the fluorescence labeled transferrin used in the NTBI assay (Breuer and Cabantchik 2001).

Increases in $O_2^{\cdot-}$ production have been observed in the PBMCs of HD with elevated serum ferritin (>600 ng/mL) after a single dose of 300 mg of IV Vitamin C. Elevated serum ferritin typically represents high stored iron in the RES but also indicates the presence of chronic inflammation in HD patients (Chen et al. 2003). Similarly, we found IS + C was associated with increased $O_2^{\cdot-}$ generation. $O_2^{\cdot-}$ is a critical mediator of intracellular signaling, thus increased generation could lead to alteration of key intracellular signaling pathways, including those promoting NFκB activation and cytokine transcription (Ogata et al. 2000). Congruent with the biologic plausibility of this pathway for cytokine activation, IS + C was associated with significant increases in serum IL-6, IL-10, TNF-α. Administration of IS alone was associated with H_2O_2 generation and a more significant loss of mitochondrial membrane potential ($\Delta\psi$m). These findings suggest that Vitamin C may exhibit both pro-oxidant and antioxidant effects in vivo and the balance of these effects is complex especially in ESRD where antioxidant systems are not intact. Other studies have found Vitamin C supplementation has either no effect or a protective effect on oxidative markers in HD patients (Fumeron et al. 2005; Shi et al. 2005; Tarng et al. 2004a, b). This may be a result of the timing of administration of Vitamin C in relation to iron. It has been shown Vitamin C protects against oxidative damage if given prior to iron, but if given after iron, then Vitamin C is more likely to enhance oxidative damage (Buettner and Moseley 1992; Otero et al. 1997).

F2-isoprostanes are derived from peroxidation of arachidonic acid by free-radicals and have been found to stimulate mitogenesis in aortic muscle cells, modulate platelet function, and are increased in human atherosclerosis (Fukunaga et al. 1993; Gniwotta et al. 1997; Morrow et al. 1992). They may be a more reliable marker of oxidative stress in HD, since they are not removed by HD. In our study, both ESRD treatment groups had in an increase in lipid peroxidation from baseline and were significantly higher than controls. Only the IS + C group produced a significant increase from baseline. This could be due to accelerating $^{\cdot}OH$ production or additional oxidation by the ascorbate radical (Buettner and Jurkiewicz 1996). Few studies have examined the effects of iron or Vitamin C supplementation on F2-isoprostanes concentration. Salahudeen et al. (2001) demonstrated that iron dextran significantly increased the levels of esterified F2-isoprostanes 30 min post-iron infusion when compared to baseline (0.199 ± 0.019 vs.

0.223 ± 0.025 ng/mL, $p < 0.01$) (Salahudeen et al. 2001). The findings among ESRD patients receiving IS + C in the current study are similar (0.198 ± 0.028 vs. 234 ± 0.04 ng/mL, $p = 0.022$). A single center study measured F2-isoprostanes during Vitamin C supplementation in HD patients found no difference in F2-isoprostanes levels after 8 weeks of 250 mg of either oral or IV Vitamin C (Chan et al. 2006). The authors did not state whether IV iron administration was allowed during the 8 week study period, therefore, lack of a pro-oxidant effect of Vitamin C in this study may be due to exclusion of IV iron during the study. However, lipid peroxidation, measured by other biomarkers (e.g. malondialdehyde, TBARS), has been reported with Vitamin C supplementation alone and when administered concomitantly with IV iron in HD patients (Eiselt et al. 2006; De Vriese et al. 2008).

Pro- and anti-inflammatory cytokines are commonly elevated in ESRD patients and CVD morbidity and mortality are correlated with oxidative stress and inflammation in CKD (Stenvinkel et al. 1999; Zimmermann et al. 1999). The etiology of acute-phase inflammation and oxidative stress in ESRD is multifactorial (Roberts et al. 2006). The role of pro-inflammatory cytokines IL-1, IL-6, and TNF-α in the development of atherosclerosis has also been confirmed in ESRD (Locatelli et al. 2003). Our results reaffirm ESRD patients have markedly higher plasma concentrations of pro- and anti-inflammatory cytokines compared to HC. In addition, we also showed the infusion of IS and IS + C induces significant inflammatory responses. These results also indicate IS + C may induce a more robust inflammatory response than IS alone.

There are several limitations of this study to consider. The principal limitation is a small sample size. Inter-patient variability in plasma cytokines is well documented in the HD population, thus confirmation of the differential effects of IV iron with and without concomitant Vitamin C is necessary in a larger population (Cohen et al. 2010). The use of a placebo arm in our study would confirm independent effects of IV iron and Vitamin C.

In summary, our study shows both IS and IS + C induce ROS, cytokine activation and lipid peroxidation in ESRD patients. Vitamin C may enhance the pro-oxidant effects of IV iron and should be avoided until larger studies address these safety concerns.

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