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Oxidative stress and renal injury with intravenous iron in patients with chronic kidney disease

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Oxidative stress and renal injury with intravenous iron in patients with chronic kidney disease.

Background. Intravenous iron is widely prescribed in patients with chronic kidney disease (CKD) and can cause oxidative stress. The relationship of oxidative stress and renal injury in patients with CKD is unknown. Whether renal injury can occur at a time point when transferrin is incompletely saturated is also unclear.

Methods. We conducted a randomized, open-label, parallel group trial to compare the oxidative stress induced by intravenous administration of 100 mg iron sucrose over 5 minutes and its protection with N-acetylcysteine (NAC) in 20 subjects with stage 3 or 4 CKD. Transferrin saturation was measured with urea polyacrylamide gel electrophoresis, oxidative stress by malondialdehyde (MDA) measurement by high-performance liquid chromatography, and renal injury by enzymuria and proteinuria. Reduced and oxidized glutathione and free radical scavengers as well as urinary monocyte chemoattractant protein-1 were also measured.

Results. Parenteral iron increased plasma concentration and urinary excretion rate of MDA, a biomarker of lipid peroxidation, within 15 to 30 minutes of iron sucrose administration. This was accompanied by enzymuria and increase in proteinuria. In contrast, saturation of transferrin was not maximally seen until 3 hours after the end of infusion. Oxidative stress, enzymuria and proteinuria were transient and were completely resolved in 24 hours. NAC reduced acute generation of systemic oxidative stress but failed to abrogate proteinuria or enzymuria.

Conclusion. Intravenous iron produces oxidative stress that is associated with transient proteinuria and tubular damage. The rapid production of oxidative stress even when transferrin is not completely saturation suggests free iron independent mechanism(s) to be operative in producing oxidative stress and transient renal injury. Long-term implications of these findings need further study.

In the United States there are approximately 8 million individuals with moderate to severe chronic kidney disease (CKD), not on dialysis [1]. Anemia sets in early during the course of renal disease, either as a result of erythropoietin deficiency or due to insufficient absorption of dietary iron [2]. Furthermore, a functional deficiency of iron may occur due to enhanced erythropoiesis after therapy with recombinant human erythropoietin that often necessitates therapy with intravenous iron [3]. The correction of anemia leads to improvement in morbidity, mortality, and quality of life [4]; however, potential toxicity due to the participation of elemental iron in oxidation-reduction reactions has raised concern of potential risks of this commonly utilized therapy [5, 6].

Oxidative stress plays an important role in the pathogenesis and progression of renal disease [7, 8]. Intravenous iron has been shown to increase biologic markers of oxidative stress [9] in cell cultures [10], animal models [11], and end-stage renal disease (ESRD) patients on hemodialysis [12–14]. Intravenous administration of iron sucrose in dialysis patients results in an increase in total peroxide, free iron, and markers of lipid peroxidation, and is significantly improved with the antioxidant, vitamin E [15]. Whether patients with CKD not on dialysis have a similar increase in oxidative stress and, above all, renal injury upon exposure to intravenous iron is unknown. This knowledge is of critical importance because oxidative stress and renal injury may lead to an accelerated course of renal [7] and cardiovascular disease [16, 17]. Furthermore, it is unknown whether the acute oxidative stress imposed by iron can be reduced with antioxidant therapy. In this context, animal and human data demonstrate beneficial effects of N-acetylcysteine (NAC) in models of oxidative stress such as contrast-induced acute renal failure [18].

We hypothesized that in subjects with moderate to severe CKD, an infusion of intravenous iron would generate oxidative stress and that this would cause tubular injury and increase in glomerular permeability. Furthermore, the generation of oxidative stress, tubular injury, glomerular permeability, and renal inflammation would be mitigated by therapy with the antioxidant NAC.

Key words: Iron, anemia, malondialdehyde, oxidative stress, chronic kidney failure, randomized controlled trial.

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METHODS

Subjects

Study participants were recruited from the Nephrology Clinic at the Richard L. Roudebush VA Medical Center. Subjects were at least 18 years in age, had an estimated glomerular filtration rate (GFR) ≤ 60 mL/min by the six-component Modified Diet in Renal Disease (MDRD) Study formula [19] but were not on renal replacement therapy and had iron deficiency anemia. Anemia was defined as blood hemoglobin concentration below the 95th percentile in the NHANES evaluation [20]. Iron deficiency was defined using National Kidney Foundation/Kidney Disease Outcome Quality Initiative (NKF-K/DOQI) guidelines as having a serum ferritin concentration of <100 ng/mL or serum transferrin saturation of <20% [21]. Subjects were excluded if they were hypersensitive to iron sucrose or NAC, were an organ transplant recipient or received therapy with immunosuppressive agents, used an investigational drug 1 month prior to study, or demonstrated the presence of active asthma, human immunodeficiency virus (HIV), cancer, rheumatoid arthritis, alcoholism, or liver disease. Subjects were also excluded if they were anemic such that erythrocyte transfusion was imminent (hemoglobin <8 g/dL, or active gastrointestinal bleeding), had acute renal failure, defined as an increase in the baseline serum creatinine concentration of ≥0.5 mg/dL over 48 hours, were pregnant or breastfeeding, had received intravenous iron within 3 months of the study, demonstrated iron overload (serum ferritin >800 ng/mL or transferrin saturation >50%), had anemia not caused by iron deficiency, had surgery or systemic or urinary tract infection within 1 month of study, or a serum albumin <3.0 g/dL.

The protocol was approved by the Institutional Review Board at Indiana University School of Medicine and the Research and Development Committee of the Richard L. Roudebush VA Medical Center. Written informed consent was obtained from each subject prior to study participation.

Study design

Subjects who satisfied the inclusion and exclusion criteria were assigned by a computer-generated randomized block randomization scheme to one of two open-label, 1-week, parallel treatment arms: either no active intervention, or treatment with the antioxidant NAC (Mucomyst) (Apothecon, Inc., Princeton, NJ, USA) at a dose of 600 mg twice a day. The allocation scheme was concealed from investigators until time of subject randomization. Oral iron supplements were stopped for the duration of the study.

The trial design is illustrated in Figure 1. Subjects were requested to be fasting before all visits. During the initial study period, baseline blood and urine was collected and assayed for biomarkers of lipid peroxidation [plasma and urine malondialdehyde (MDA)], substrate for lipid peroxidation (lipid panel), evaluation of iron deficiency anemia [complete blood count, iron, total iron binding capacity (TIBC), ferritin], redox state [plasma and erythrocyte oxidized and reduced glutathione (GSSG, GSH)], free radical scavengers [erythrocyte superoxide dismutase (red blood cell SOD) and glutathione peroxidase (GSHPx)], and markers of renal tubular and glomerular damage and/or inflammation [urinary monocyte chemoattractant protein-1 (MCP-1), urine total protein, and N-acetyl-β-D-glucosaminidase (NAG)]. Subjects then received an intravenous dose of iron sucrose (Venofer) (American Regent Laboratories, Inc., Shirley, NY, USA) at a dose of 100 mg infused over 5 minutes. Blood and urine collections were obtained at baseline, 0.25, 0.5, 1, 2, 3, and 24 hours after iron dosing to assay for the above parameters. During the visits, the subjects completed the Kidney Disease and Quality of Life Short Form (KDQOL-SF, version 1.3). Subjects were randomized to take either no additional treatment or NAC 600 mg twice a day for 1 week. The last dose of NAC was administered under direct supervision by the study nurse in the morning of the second infusion of intravenous iron. After 1 week, subjects had all study procedures repeated as on the initial study period. NAC-assigned subjects returned with remaining antioxidant doses to assess compliance. Subjects completed a KDQOL-SF modified to assess parameters over the 1-week intervening treatment period.

Laboratory analysis

Electrophoretic separation of transferrins. The iron forms of transferrin were separated using a urea polyacrylamide gel (6% acrylamide gels with 6 mol/L urea) according to Williams, Evans, and Moreton [22]. This method separates transferrin into the apotransferrin, monoferric, and the diferric forms, according to their electrophoretic mobilities. Precipitation of transferrin from plasma was achieved through treatment with 2% 6,9-diamino-2-ethoxyacridine lactate monohydrate (Sigma Chemical Co., St. Louis, MO, USA) and subsequent centrifugation before being applied to the gel to precipitate all serum proteins but β and γ globulins [23]. Electrophoresis was performed using a Criterion mini-gel system (Bio-Rad, Hercules, CA, USA). Fourteen plasma samples obtained from each subject over two visits were separated simultaneously on the same gel, along with controls of apotransferrin, monoferric transferrin(s), and diferric transferrin. Protein bands were visualized through staining with GelCode Blue stain reagent (Pierce, Rockford, IL, USA). Densitometric analysis was performed with a Gel Logic 100 apparatus and one-dimensional image analysis software (Kodak, Rochester,

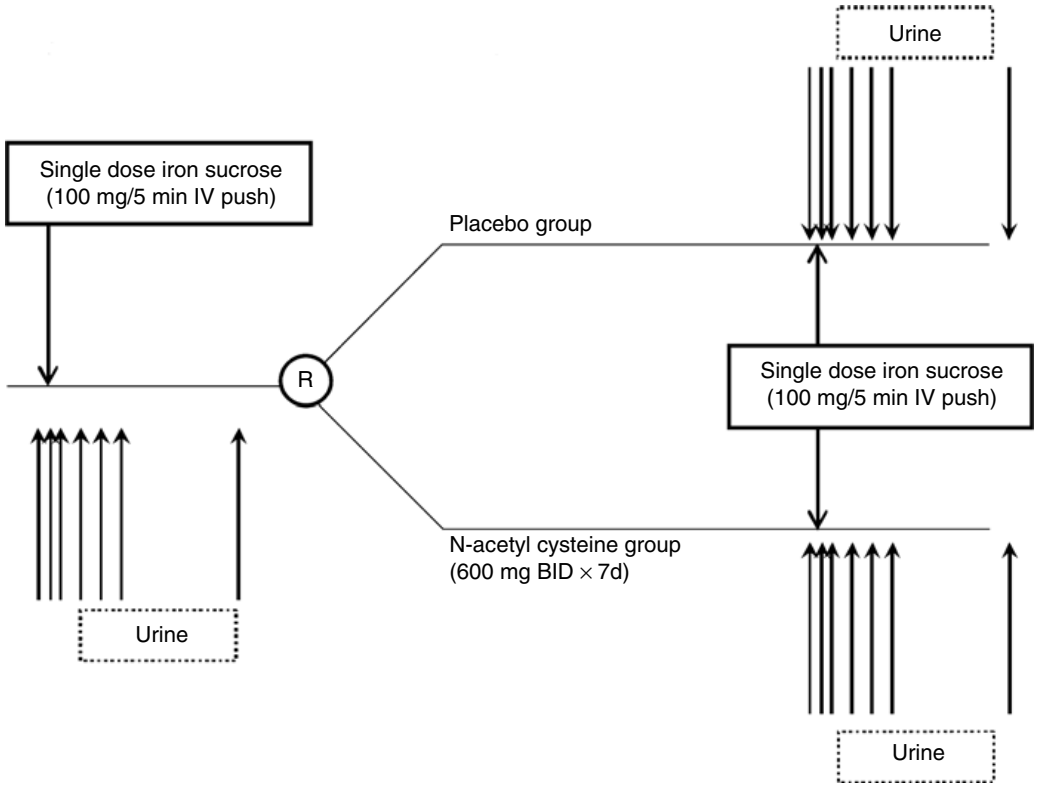

Fig. 1. Trial design. All patients with chronic kidney disease (CKD) had baseline urine and blood draws followed by administration of iron sucrose. Blood and urine specimens were collected at 0.25, 0.5, 1, 2, 3, and 24 hours after administration of the drug. Patients were then randomized to receive N-acetylcysteine (NAC) or nothing for 1 week in an open-label fashion. After 1 week, iron sucrose was again administered and tests performed as done at first visit.

NY, USA). Percent transferrin saturation was calculated as follows:

$$\text{Transferrin Saturation}(\%) = \frac{[\text{Diferric transferrin} + (1/2 \times \text{monoferric transferrin(s)})]}{[\text{Apotransferrin} + \text{Monoferric transferrin(s)} + \text{Diferric transferrin}]}$$

Reduced and oxidized glutathione. Determination of reduced and oxidized glutathione concentrations was achieved through an isocratic high-performance liquid chromatography (HPLC) technique. Specimens were collected, processed, and analyzed as described by Paroni et al [24]. We modified the method by increasing the flow rate and changing the wash pattern of the column as described below.

Briefly, the mobile phase consisted of 21 mmol/L proprionate buffer and acetonitrile, 95:5 (vol/vol) pumped at 2 mL/min. The column was a Merck LiChrospher RP-18, 5 μ with guard placed in a column warmer set to 37°C. Following three consecutive samples, the column was washed for 3 minutes with a solution of acetonitrile:methanol:water (55:25:20 vol/vol) followed by re-equilibration with mobile phase for 4 minutes. The chromatographic system consisted of a Hewlett-Packard Chromatographic Series 1100 autosampler and isocratic pump and Hewlett-Packard model 1046A programmable fluorescence detector (Palo Alto, CA, USA). The autosampler was programmed to perform precolumn derivatization with *o*-phthalaldehyde with peak detection by fluorescence with an excitation wavelength of 340 nm and an emission wavelength 420 nm. Peak areas were determined using a ChromJet integrator (Thermo Separation Products, San Jose, CA, USA).

Plasma and urinary MDA assay. MDA, a lipid hydroperoxide, is formed by β-scission of peroxidized polyunsaturated fatty acids was measured by derivatization with thiobarbituric acid (TBA) as reported previously [25]. Briefly, the mobile phase consisted of 40:60 ratio (vol/vol) of methanol to 50 mmol/L potassium monobasic phosphate at pH 6.8, pumped at a rate of 1.0 mL/min on a Hewlett-Packard Hypersil 5 μ ODS 100 × 4.6 mm placed in a column warmer set to 37°C. Samples of plasma and urine were treated with the antioxidant, butylated hydroxytoluene (to prevent in vitro oxidation) and heat derivatized at 100°C for 1 hour with

TBA at an acid pH. Samples were extracted with n-butanol and 10 μL of the extract was injected at 1-minute intervals using an autosampler. The Hewlett-Packard model 1046A programmable fluorescence detector was set at excitation of 515 nm and emission of 553 nm. Retention time was 1.87 minutes; however, absence of interfering peaks, allows analysis to be carried out in increments of 1 minute per sample. Within-day variability in estimation was between 8.6% and 10.3%. Between-days variability was 3.6% and 7.9%. Recovery was between 88% and 101%.

Urinary MCP-1 assay. MCP-1 was assayed in urine using a sandwich ELISA (Quantikine® kit for Human MCP-1 Immunoassay) (R&D Systems, Minneapolis, MN, USA). A standard curve was generated using a four parameter logistic curve-fit. Corrections were made for concentration and values were expressed as pg MCP-1 per mg creatinine.

SOD and GSHPx activity. SOD activity was measured in red blood cell lysate using an end point spectrophotometric technique (Superoxide Dismutase Assay) (Cayman Chemical Co., Ann Arbor, MI, USA). GSHPx activity in red blood cell lysate was determined using an indirectly coupled reaction with glutathione reductase in a kinetic spectrophotometric assay (Glutathione Peroxidase Assay) Cayman Chemical Co.).

Urine protein, creatinine, and NAG determination. Concentration of urinary protein was quantified through an end point spectrophotometric assay utilizing pyrogallol red-molybdate (quanTtest red) (Quantimetrix, Redondo Beach, CA, USA). Urine creatinine concentration was determined using an end point spectrophotometric with an alkaline-picrate solution (Creatinine Kit) (Sigma Diagnostics, St. Louis, MO, USA). Urinary NAG was measured by colorimetric assay (Roche Diagnostics Corporation, Indianapolis, IN, USA). Other laboratory assays were performed using standard methods in the hospital laboratory.

Statistical analysis

The primary outcome variable of this study was the time course of urinary MDA, protein and NAG excretion rates, total amount excreted and their relationship to serum transferrin saturation. Other outcome variables included the influence of NAC on these parameters. Treatment effects were analyzed using repeated measures analysis of variance with time and treatment as independent factors. Differences between means was tested by the least significant difference test. Scales of KDQOL were analyzed by repeated measures, one-way analysis of variance (ANOVA). All P values are two sided and significance set at <0.05. All statistical analyses were performed using Statistica for Windows, version 5.5 (StatSoft, Inc., Tulsa, OK, USA).

Table 1. Baseline characteristics of the study population

	Iron sucrose + placebo	Iron sucrose + N-acetylcysteine (NAC)	Overall
Number of patients	10	10	20
Age *years*	71 ± 5.9	75 ± 7.6	73 ± 7.0
Male *number*	10	10	20
Whites/Blacks *number*	8/2	7/3	15/5
Weight *kg*	93 ± 19.2	97 ± 23.8	96 ± 21.3
Body mass index $kg \cdot m^2$	30 ± 12.5	32 ± 7.6	31 ± 10.1
Tobacco use *number*			
Current	2	2	4
Former	9	8	17
Coronary artery disease *number*	6	8	14
Etiology of kidney disease *number*			
Hypertension	3	3	6
Diabetes mellitus	6	6	12
Other	0	1	1
Angiotensin-convering enzyme (ACE) inhibitor therapy *number*	3	7	10
Systolic blood pressure *mm Hg*	138 ± 29.0	148 ± 23.4	143 ± 26.1
Diastolic blood pressure *mm Hg*	69 ± 10.5	66 ± 11.7	67 ± 11.0
Heart rate *beats/min*	70 ± 10.1	64 ± 10.7	67 ± 10.6
Modification of Diet in Renal Disease Study glomerular filtration rate *mL/min*	25 ± 7.1	27 ± 7.4	26 ± 7.2
Angiotensin receptor blocker therapy *number*	5	5	10
Erythropoietin therapy *number*	4	2	6
Hemoglobin *g/dL*	11.3 ± 1.1	11.1 ± 0.7	11.2 ± 0.9
Iron μg/dL	60.3 ± 28.8	59.4 ± 19.0	60.3 ± 28.8
Total iron binding capacity μg/dL	351 ± 92	322 ± 67	336 ± 80
Ferritin *ng/mL*	85 ± 75	127 ± 100	106 ± 89
Serum albumin g/dL	3.8 ± 0.4	4.0 ± 0.3	4.0 ± 0.4
Total cholesterol *mg/dL*	151 ± 53.5	160 ± 54.2	155 ± 52.6
Low-density lipoprotein cholesterol *mg/dL*	80 ± 37.1	74 ± 25.7	77 ± 31.2
High-density lipoprotein cholesterol *mg/dL*	38 ± 11.8	39 ± 8.3	38 ± 9.9
Triglycerides *mg/dL*	178 ± 112.4	224 ± 155.2	201 ± 134.0

RESULTS

Study participants

Actual subject study participation occurred between April 2003 and August 2003. Baseline and clinical characteristics are shown in Table 1. Subjects treated with intravenous iron with and without NAC were similar with respect to demographic and clinical characteristics outlined. There were no women reflecting the paucity of women with kidney disease among the veteran population. The distribution of the etiology of kidney disease, age, race, weight, and body mass index were similar. Baseline hematologic, lipid, and other laboratory characteristics were well matched.

There was no increase in hemoglobin or reticulocytes over 1 week (Table 2). In fact, there was a small fall

Table 2. Baseline laboratory parameters before and after treatment with N-acetylcysteine (NAC) or no antioxidants

	Iron sucrose + placebo		Iron sucrose + NAC	
	Pre	Post	Pre	Post
White blood cells ×1000/μL	8.0 ± 3.6	7.8 ± 3.4	7.3 ± 1.9	6.7 ± 2.0
Hemoglobin g/dL	11.3 ± 1.1	11.3 ± 0.9	11.1 ± 0.7	10.8 ± 0.5
Mean corpuscular volume fL	84.0 ± 6.2	83.9 ± 6.7	89.5 ± 5.6	89.3 ± 5.5
RDW %	15.9 ± 1.6	15.8 ± 1.3	14.6 ± 1.6	14.5 ± 1.7
MCHC g/dL	33.5 ± 0.7	33.8 ± 0.7	34.1 ± 0.8	34.1 ± 0.8
Platelets ×1000/μL	237 ± 95.6	243 ± 91.4	198 ± 40.6	192 ± 38.7
Reticulocytes %	1.9 ± 0.5	1.8 ± 0.3	1.4 ± 0.6	1.4 ± 0.4
Iron μg/dL	59.4 ±28.8	51.9 ±14.7	59.4 ±19.0	54.1 ± 12.8
Total iron binding capacity μg/dL	351 ± 92.4	368 ± 91.9	322 ± 67.1	317 ± 44.2
Transferrrin saturation %	19 ± 6	18 ± 10	17 ± 3	15 ± 5
Ferritin ng/μL	85 ± 75.4	91 ± 57.2	127 ± 99.9	169 ± 107.3

Abbreviations are: RDW, red cell distribution width; and MCHC, mean corpuscular hemoglobin concentration. Data shown are mean ± standard deviation. None of the changes are statistically significant except in hemoglobin (fall of 0.26 g/dL, (95% CI 0.5 to 0, $P = 0.042$) and in serum ferritin (increase of 30.3 ng/mL, 95% CI 17.9 to 42.6 ng/mL, $P < 0.001$).

in hemoglobin concentration of 0.26 g/dL ($P = 0.042$). Serum iron and TIBC were unchanged over 1 week, but serum ferritin increased by 30.3 ng/mL ($P < 0.001$).

Time course of transferrin saturation, generation of oxidative stress, and renal injury

Figure 2 shows the overall results of the trial. Plasma and urinary MDA (top two rows of plots) increased rapidly within 30 minutes of administration of intravenous iron and were accompanied by an increase in proteinuria and enzymuria (third and fourth rows of plots). In contrast, transferrin saturation did not peak until 3 hours and returned to baseline by 24 hours (last row of plots). A significant effect of time was seen for all variables shown in Figure 1. Plasma MDA was increased at the 15 and 30 minutes time point and was significantly different from the remaining levels (ANOVA, $P < 0.0001$). Urine MDA and NAG were maximal at 30 minutes and significantly different from rest of the excretion rates (ANOVA, $P < 0.0001$). Proteinuria was also maximal at 30 minutes but the excretion rates at 15 and 60 minutes collection points were also similar to that at 30 minutes (ANOVA, $P < 0.0001$). Since there was no effect of NAC and no difference between the two visits, least square means from ANOVA and pooled standard errors of the means are shown in Table 3 to illustrate the time course of the oxidative stress, iron saturation, and response.

Effect of NAC on generation of oxidative stress and renal injury

Compliance with intake of NAC assessed by measuring the volume of returned drug was 101% ± 8%. Because oxidative stress occurred within minutes, the area under the curve (AUC) of the plasma MDA concentration time curves were compared from baseline to 180 minutes. There was a significant improvement in plasma MDA generation with NAC ($P = 0.048$). The AUC between the two intravenous iron infusions using placebo were 184 ± 244 μmol × min/L and 244 ± 51 μmol × min/L, whereas with NAC they were 217 ± 60 μmol × min/L and 199 ± 45 μmol × min/L, respectively (Fig. 3, top panels). The redox ratio measured by plasma oxidized to reduced glutathione remained unaltered with treatment with NAC (Table 4). The total amount of MDA, protein, and NAC excreted in urine over the 24 hours in patients treated with NAC and placebo were unchanged. Although NAC did not change the 24-hour or the first 3-hour excretion of urinary MDA, Figure 3 shows that the amount of MDA excreted from 3 to 24 hours was reduced with NAC from 4.24 ± 2.02 μg to 3.36 ± 1.47 μg compared to 3.50 ± 1.19 μg to 4.08 ± 1.88 μg following placebo (ANOVA for interaction, $P = 0.041$). The free radical scavenging enzymes in the red blood cells such as SOD and GSHPx remained unchanged with the administration of NAC (Table 4). NAC was unable to reduce the urinary MCP-1 levels that were unchanged 1 week after intravenous iron administration (Table 4).

Changes in kidney disease quality of life

There were no statistically significant changes in KDQOL, except in the pain scale (Table 5). Despite needle sticks and intravenous catheters, subjects reported improvement in pain (higher number is associated with less pain) on average 14.5% (95% CI, 0.06 to 28.9, $P = 0.049$). A trend toward improvement in physical functioning was seen 5.25% (95% CI, −1.1 to 11.6, $P = 0.10$).

Safety and adverse events

There were two serious adverse events seen. One patient had sudden death the night after the day of infusion. He had underlying coronary artery disease and the event was not believed to be related to the study drug. Two patients had diarrhea thought to be related to the study drug. One patient had diarrhea about 12 hours after the infusion that was accompanied by bloody stools. The other patient had diarrhea within 1 hour of administration of the study drug.

DISCUSSION

The major findings of our study are that oxidative stress occurs rapidly with infusion of intravenous iron sucrose in patients with CKD in doses recommended by the manufacturer. Oxidative stress so induced is accompanied by the occurrence of renal tubular damage and possibly increase in glomerular permeability as assessed by enzymuria and increase in proteinuria, respectively. These

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