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In vitro and *in vivo* evidence suggesting a role for iron in cisplatin-induced nephrotoxicity

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***In vitro* and *in vivo* evidence suggesting a role for iron in cisplatin-induced nephrotoxicity.** Cisplatin is a widely used antineoplastic agent that has nephrotoxicity as a major side effect. The underlying mechanism of this nephrotoxicity is still not well known. Iron has been implicated to play an important role in several models of tissue injury, presumably through the generation of hydroxyl radicals via the Haber-Weiss reaction or other highly toxic free radicals. In the present study we examined the catalytic iron content and the effect of iron chelators in an *in vitro* model of cisplatin-induced cytotoxicity in LLC-PK_1 cells (renal tubular epithelial cells) and in an *in vivo* model of cisplatin-induced acute renal failure in rats. Exposure of LLC-PK_1 cells to cisplatin resulted in a significant increase in bleomycin-detectable iron (iron capable of catalyzing free radical reactions) released into the medium. Concurrent incubation of LLC-PK_1 cells with iron chelators including deferoxamine and 1,10-phenanthroline significantly attenuated cisplatin-induced cytotoxicity as measured by lactate dehydrogenase (LDH) release. Bleomycin-detectable iron content was also markedly increased in the kidney of rats treated with cisplatin. Similarly, administration of deferoxamine in rats provided marked functional (as measured by blood urea nitrogen and creatinine) and histological protection against cisplatin-induced acute renal failure. In a separate study, we examined the role of hydroxyl radical in cisplatin-induced nephrotoxicity. Incubation of LLC-PK_1 cells with cisplatin caused an increase in hydroxyl radical formation. Hydroxyl radical scavengers, dimethyl sulfoxide, mannitol and benzoic acid, significantly reduced cisplatin-induced cytotoxicity and, treatment with dimethyl sulfoxide or dimethylthiourea provided significant protection against cisplatin-induced acute renal failure. Taken together, our data strongly support a critical role for iron in mediating tissue injury via hydroxyl radical (or a similar oxidant) in this model of nephrotoxicity.

Cisplatin is one of the most effective chemotherapeutic agents and plays a major role in the treatment of a variety of human solid tumors [1]. The most common adverse effect limiting the efficacy of the antineoplastic agent is nephrotoxicity, which develops primarily in the S3 segment of the proximal tubule [2]. Aggressive hydration and forced diuresis is utilized to reduce cisplatin nephrotoxicity [3]. The mechanism underlying this nephrotoxicity is not well understood [4].

Key words: iron in nephrotoxicity, nephrotoxicity, cisplatin-induced nephrotoxicity, antineoplastic agent, hydroxyl radicals, tissue injury.

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Recent studies indicate an important role of iron in tissue injury involving reactive oxygen species [5]. The ease with which iron is reversibly oxidized and reduced makes iron potentially hazardous because of its ability to participate in the generation of powerful oxidant species, such as the hydroxyl radical via the metal catalyzed Haber-Weiss reaction and/or in the generation of the highly reactive iron-oxygen complexes such as ferryl or perferryl ions [5]. *In vivo* most of the iron is bound to heme and non-heme proteins, and does not directly catalyze the generation of hydroxyl radicals or a similar oxidant [5]. Gutteridge et al have described an assay, based on the use of the antibiotic, bleomycin, to detect iron complexes capable of catalyzing free radical reactions in biological samples [6]. Using this assay, an increase in iron capable of catalyzing free radical reactions has been demonstrated in ischemia/reperfusion-induced acute renal failure [7], and in glycerol-induced acute renal failure [8]. Iron chelators, including deferoxamine and 2,3-dihydroxybenzoic acid, have been shown to be protective in several models of acute renal failure [9–11] and oxidant-induced cell injury [12, 13]. The role of iron in cisplatin-induced nephrotoxicity has not been examined previously.

The major purpose of the current study was to examine the role of catalytic iron and hydroxyl radical in an *in vitro* model of cisplatin-induced cytotoxicity in LLC-PK_1 cells (renal tubular epithelial cells) and in an *in vivo* model of cisplatin-induced acute renal failure in rats.

METHODS

In vitro study

Cell culture. LLC-PK_1 cells (renal proximal tubular epithelial cells), purchased from American Type Culture Collection (CRL 1392), were maintained in Medium 199 supplemented with 3% fetal bovine serum and penicillin (100 U/ml) in a humidified atmosphere of 95% air—5% CO_2. The cells were maintained in 75 cm^2 tissue culture flask and the monolayers were subcultured using 0.05% trypsin—0.53 mM EDTA in calcium- and magnesium-free Hank's balanced salt solution (HBSS). For the experimental study, the cells were grown in a 6- or 12-well tissue culture plate until confluency. All experiments were carried on confluent cell monolayer between passages 203-215.

Cisplatin-induced cytotoxicity. On the day of the experiment, the medium was discarded and the confluent LLC-PK_1 cell monolayer was washed twice with Hank's balanced salt solution (HBSS). The cells were then incubated with various concentrations of cisplatin (0 to 500 μg/ml) for different periods of time (0 to 5 hr) in HBSS

at 37°C. The dose of cisplatin used in the study is based on previous studies [14, 15]. At the end of the incubation, the incubation medium was collected and the remaining cell monolayer was dissolved in 0.1% Triton X-100. Lactate dehydrogenase (LDH) in the medium and in the cellular fraction was measured by the use of an assay kit purchased from Sigma Chemical Co. (St. Louis, MO, USA). The degree of cell injury induced by cisplatin was expressed as LDH release which is the percentage of LDH released into the medium to total LDH recovered from both medium and cellular fractions.

Effect of cisplatin on catalytic iron released. Confluent cell monolayer was washed three times with Chelex-treated HBSS to remove as much contaminating iron as possible. The cells were then incubated with a cytotoxic dose of cisplatin in Chelex-treated HBSS at 37°C for a period of time just before substantial cell killing occurred. At the end of incubation, the incubation medium was collected for the measurement of catalytic iron released by bleomycin assay as described below.

Effect of cisplatin on hydroxyl radical formation. Confluent cell monolayer was washed three times with HBSS and then incubated with cytotoxic dose of cisplatin in HBSS at 37°C for a period of time before substantial cell killing occurred. 2-Deoxy-D-ribose in a final concentration of 3 mM was added to the medium just prior to the incubation. At the end of incubation, the incubation medium was collected for the measurement of hydroxyl radical formation by deoxyribose degradation method described by Halliwell, Grootveld and Gutteridge [16]. In brief, 0.5 ml of incubation medium was mixed with 0.5 ml of 1% (wt/vol) solution of thiobarbituric acid in 50 mM NaOH and 0.5 ml of 2.8% (wt/vol) aqueous trichloroacetic acid, heated at 100°C for 15 minutes, cooled, and followed by extraction with 1.5 ml *n*-butanol. The resulting supernatant was then read at 532 nm and the amount of hydroxyl radical formation was calculated by using the extinction coefficient of 156 mM^{-1} cm^{-1}.

Effect of iron chelators and hydroxyl radical scavengers on cisplatin-induced cytotoxicity. Confluent cell monolayer was washed out of medium and then incubated with a cytotoxic dose of cisplatin in HBSS at 37°C for a period of time necessary to induce consistent cytotoxicity. Iron chelators, deferoxamine and 1,10-phenanthroline, and hydroxyl radical scavengers, dimethyl sulfoxide, mannitol and benzoic acid, were used to increase the reproducibility. Deferoxamine (DFO; 1 mM), 1,10-phenanthroline (0.1 mM), dimethyl sulfoxide (DMSO; 5 mM), mannitol (50 mM) or benzoic acid (100 mM) was added to the incubation medium just prior to the addition of cisplatin. Lactate dehydrogenase (LDH) release was measured at the end of the incubation.

Effect of iron chelator on platinum level. To exclude the possibility that an iron chelator may interfere with the platinum level of cisplatin, cisplatin at a dose to induce consistent cytotoxicity (200 μg/ml) was incubated with or without the iron chelator, deferoxamine, in a range of 1 to 5 mM and then the platinum level was measured by atomic absorption spectroscopy with electrothermal atomization. The assay was performed by Dr. Thomas Moyer in the Department of Laboratory Medicine and Pathology at Mayo Clinic in Rochester, Minnesota.

In vivo study

Cisplatin-induced acute renal failure. The cisplatin model of acute renal failure was induced in rats as described by Capasso et al [17]. Male Sprague-Dawley rats weighing 200 to 250 g were allowed free access to rat chow (Purina) and water. The animals received intraperitoneal injection of cisplatin (Bristol-Myers Squibb Co., Princeton, NJ, USA) at a dose of 10 mg/kg body wt and were sacrificed 72 hours after cisplatin injection. Blood and kidneys were obtained for the various measurements. Renal impairment was assessed by blood urea nitrogen and plasma creatinine as well as kidney histology. Blood urea nitrogen (BUN) and creatinine were measured by the use of Sigma diagnostic kits. Kidney histology was studied as described in the following section.

Effect of iron chelator and hydroxyl radical scavengers on cisplatin model of acute renal failure. The iron chelator, DFO and hydroxyl radical scavengers, DMSO or dimethylthiourea (DMTU), were used in separate experiments with rats receiving cisplatin treatment. DFO was administered via an osmotic pump (type 2 ML-1; ALZA Corp. Palo Alto, CA) that was implanted subcutaneously 24 hours prior to cisplatin injection until sacrifice. The drug was reconstituted in water at a concentration of 125 mg/ml, and the pumps (with a 2 ml capacity) delivered approximately 30 mg DFO per rat per day at a continuous rate of 10 μl/hr [9, 11]. DMSO was administered via intraperitoneal injection twice a day at a dose of 4 g/kg body wt until sacrifice [9]. The first dose of DMSO was given four hours prior to cisplatin injection. DMTU and urea (which is not a hydroxyl radical scavenger and serves as a control) were administered via intraperitoneal injection 30 minutes prior to cisplatin injection at a dose of 500 mg/kg body wt, followed by twice a day at a dose of 125 mg/kg body wt until sacrifice [9].

Kidney histology. The kidneys were sectioned and a portion fixed in 10% formalin, dehydrated and embedded in paraffin. Sections were cut at 2 μm and stained with periodic acid-Schiff reagent. The slides were coded and semiquantitative analysis of the kidney sections was performed without knowledge of the treatment protocol. The changes seen were limited to the tubulointerstitial areas and graded as follows: 0, normal; I, areas of tubular epithelial cell swelling, vacuolar degeneration, necrosis, and desquamation involving $<25\%$ of cortical tubules; II, similar changes involving $>25\%$ but $<50\%$ of cortical tubules; III, similar changes involving $>50\%$ but $<75\%$ of cortical tubules; IV, similar changes involving $>75\%$ of cortical tubules [18].

Bleomycin-detectable iron assay. Catalytic iron (capable of catalyzing free radical reactions) released into the culture medium or present in kidney was measured by the bleomycin assay developed as detailed in our previous study [7]. For the measurement of iron in the kidney, tissues were homogenized in Chelex-treated pyrogen-free water, loaded into a Centriprep-30 which has a cut-off value of 30,000 kDa, and centrifuged at 4°C for 30 minutes at 1500 g. The clear ultrafiltrate was collected and used for bleomycin-detectable iron determination.

One milliliter of the reaction mixture contained in order: 0.5 ml of calf thymus DNA (1 mg/ml), 0.05 or 0.1 ml of bleomycin sulfate (1 mg/ml), 0.1 ml of $MgCl_2$ (50 mM), 0.1 ml of sample, 0.1 ml of Chelex-treated pyrogen-free water, 0.1 ml of ascorbic acid (8 mM), and either 0.05 ml of HCl (10 mM) [7] or 0.1 ml of imidazole (1.0 M, pH 7.3) [19] to adjust to pH 7.4. Sample blanks were identical except that bleomycin was omitted. The samples were then incubated at 37°C for two hours with shaking. The reaction was stopped by adding 0.1 M EDTA, and mixed with 1 ml of thiobarbituric acid (1% wt/vol in 50 mM NaOH) and 1 ml of 25% HCl (vol/vol). The reaction mixture was then heated at 100°C for 15 minutes, cooled, and the resulting chromogen measured using spectrophotometer by its absorbance at 532 nm. A standard curve

was prepared using known amounts of $FeCl_3$ in Chelex-treated pyrogen-free water. The amount of bleomycin-detectable iron in the test sample was calculated from the standard curve and the results expressed as either nmol/mg cellular protein recovered from the cell monolayer or nmol/mg tissue protein recovered from the homogenate. Protein was measured by the use of Bio-Rad reagent. All reagents except for the sample under test, were made up in Chelex-treated pyrogen-free water and shaken with Chelex-100 to remove as much contaminating iron as possible.

Statistical analysis

Values are expressed as mean ± standard error (SE). Statistical analyses were performed using unpaired *t*-test (for only two groups) and analysis of variance (for more than two groups). Statistical significance was considered at $P < 0.05$.

RESULTS

We first examined the time course and dose-dependent effect of cisplatin on irreversible cell death in LLC-PK_1 cells as measured by LDH release. As shown in Figure 1A, cisplatin-induced cell death to LLC-PK_1 cells was first observed with a dose of 50 μg/ml with increasing cytotoxicity with the highest dosage tested of 500 μg/ml. Based on the concentration-dependent effect of cisplatin at four hours, we chose the concentration, 200 μg/ml of cisplatin, which was the dose required to cause submaximal injury over a two hour period. Exposure of LLC-PK_1 cells to cisplatin at a cytotoxic dose (200 μg/ml) resulted in significant increase in LDH release at three hours with increases up to five hours (Fig. 1B).

LLC-PK_1 cells incubated for 2.5 hours without cisplatin had 1.06 ± 0.27 nmol/mg protein catalytic iron (as measured by bleomycin-detectable iron assay) in the medium. Exposure of LLC-PK_1 cells to cisplatin resulted in a marked increase (5.86 ± 0.46 nmol/mg protein, $N = 4$, $P < 0.01$) in catalytic iron released into the medium (Fig. 2). We have previously shown that catalytic iron does not increase nonspecifically as a result of cell injury or in homogenized tissue [7]. In separate experiments we examined the effect of metal chelators, DFO and 1,10-phenanthroline, on cisplatin-induced cytotoxicity in LLC-PK_1 cells (Fig. 3). LLC-PK_1 cells incubated with 200 μg/ml of cisplatin for four hours resulted in 78 ± 2% LDH release compared to control value of 5 ± 1% ($N = 4$, $P < 0.01$). Both DFO and 1,10-phenanthroline completely prevented the cytotoxicity with LDH release being reduced to 3.3 ± 0.7% and 3.0 ± 0.3%, respectively. These concentrations of DFO and 1,10-phenanthroline also prevented increases in the bleomycin-detectable iron (data not shown).

In an *in vivo* study we examined the catalytic iron in the kidney in a model of cisplatin-induced acute renal failure. Cisplatin at a dose of 10 mg/kg intraperitoneally resulted in acute renal failure (Fig. 4 A, B). The bleomycin-detectable iron content in the kidney was significantly increased from a control value of 0.85 ± 0.06 to 1.7 ± 0.09 nmol/mg protein ($N = 5$, $P < 0.01$) in rats treated with cisplatin (Fig. 4C). We examined the effect of DFO on cisplatin-induced acute renal failure. Rats treated with cisplatin and concurrently treated with DFO had significantly less deterioration in renal function (BUN, 66 ± 13 mg/dl; creatinine, 1.50 ± 0.28 mg/dl; $N = 10$, $P < 0.01$) compared to rats receiving cisplatin alone (BUN, 109 ± 6 mg/dl; creatinine, 2.58 ± 0.19 mg/dl; $N = 10$, $P < 0.01$; Fig. 5). In a separate study utilizing a cell-free system, we confirmed that the level of cisplatin measured with and without an iron chelator, DFO was similar, indicating that the protective

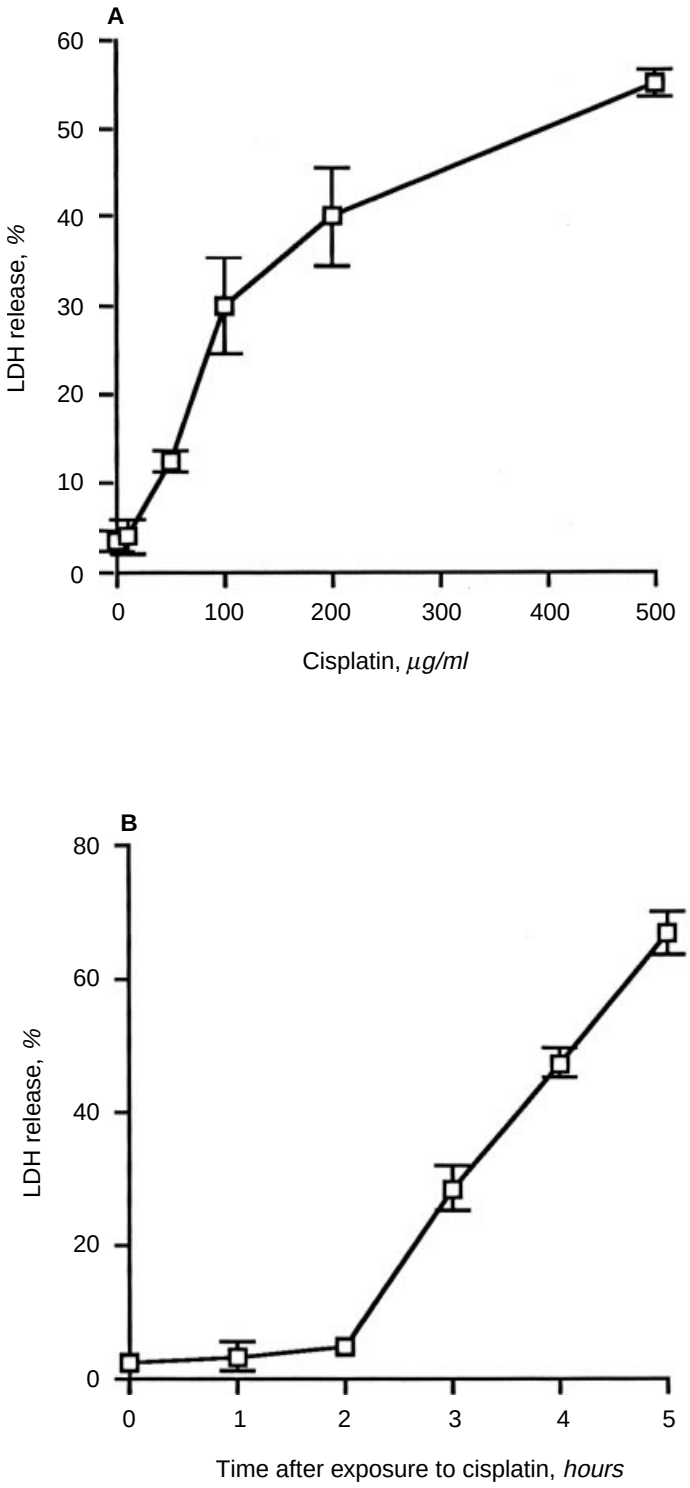

Fig. 1. Concentration-dependent and time-dependent effect of cisplatin on cytotoxicity in LLC-PK_1 cells. Confluent cell monolayer was incubated with various concentrations of cisplatin for different periods of time as indicated. Lactate dehydrogenase (LDH) release was measured at the end of incubation. (*A*) Concentration-dependent effect of cisplatin (0 to 500 μg/ml, 4 hr) on cytotoxicity as measured by LDH release in LLC-PK_1 cells. (*B*) Time course of cisplatin (200 μg/ml, 0 to 5 hr) on cytotoxicity as measured by LDH release in LLC-PK_1 cells. Values are means ± SE, $N = 2$.

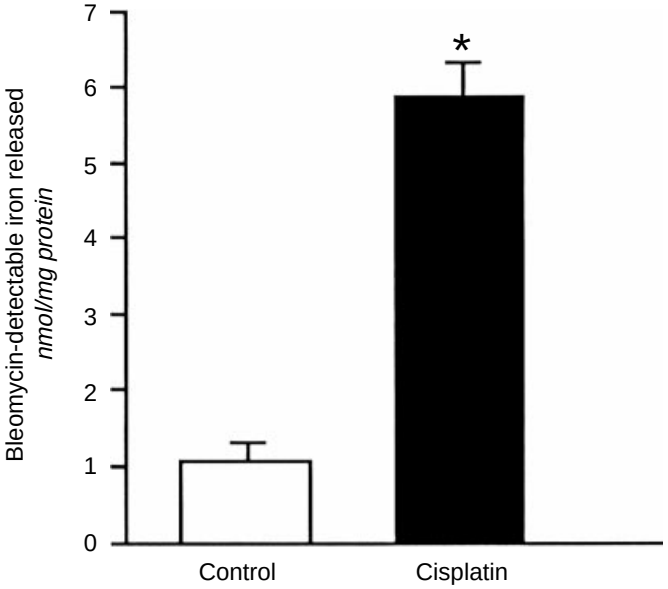

Fig. 2. Effect of cisplatin on catalytic iron released as measured by bleomycin-detectable iron in LLC-PK$_1$ cells. Confluent cell monolayer was washed with Chelex-treated Hank's balanced salt solution (HBSS) to remove contaminating iron and then incubated with 200 μg/ml of cisplatin for 2.5 hours in Chelex-treated HBSS. At the end of the incubation, the incubation buffer was collected for the measurement of bleomycin-detectable iron as described in the **Methods** section. Values are means ± SE, $N = 4$, $^*P < 0.01$, compared with control cells.

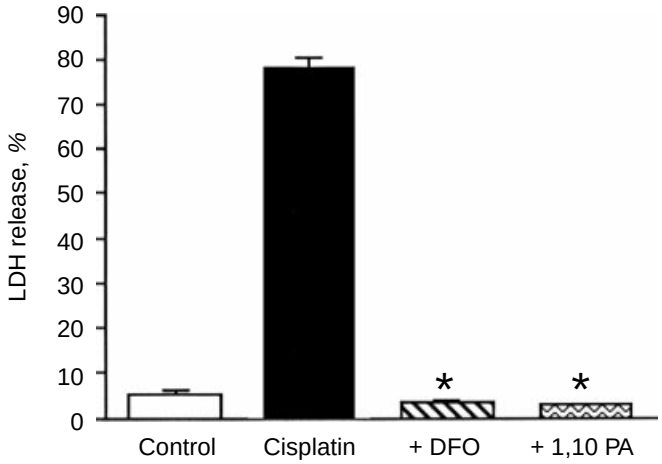

Fig. 3. Effect of iron chelators on cisplatin-induced cytotoxicity as measured by lactate dehydrogenase (LDH) release in LLC-PK$_1$ cells. Confluent cell monolayer was incubated with 200 μg/ml of cisplatin for four hours. Iron chelators including deferoxamine (DFO, 1 mM) and 1,10-phenanthroline (1,10 PA, 0.1 mM) were added to the medium just prior to the addition of cisplatin. LDH release was measured at the end of incubation as described in the **Methods** section. Values are means ± SE. $N = 4$, $^*P < 0.01$, compared with cisplatin treatment alone.

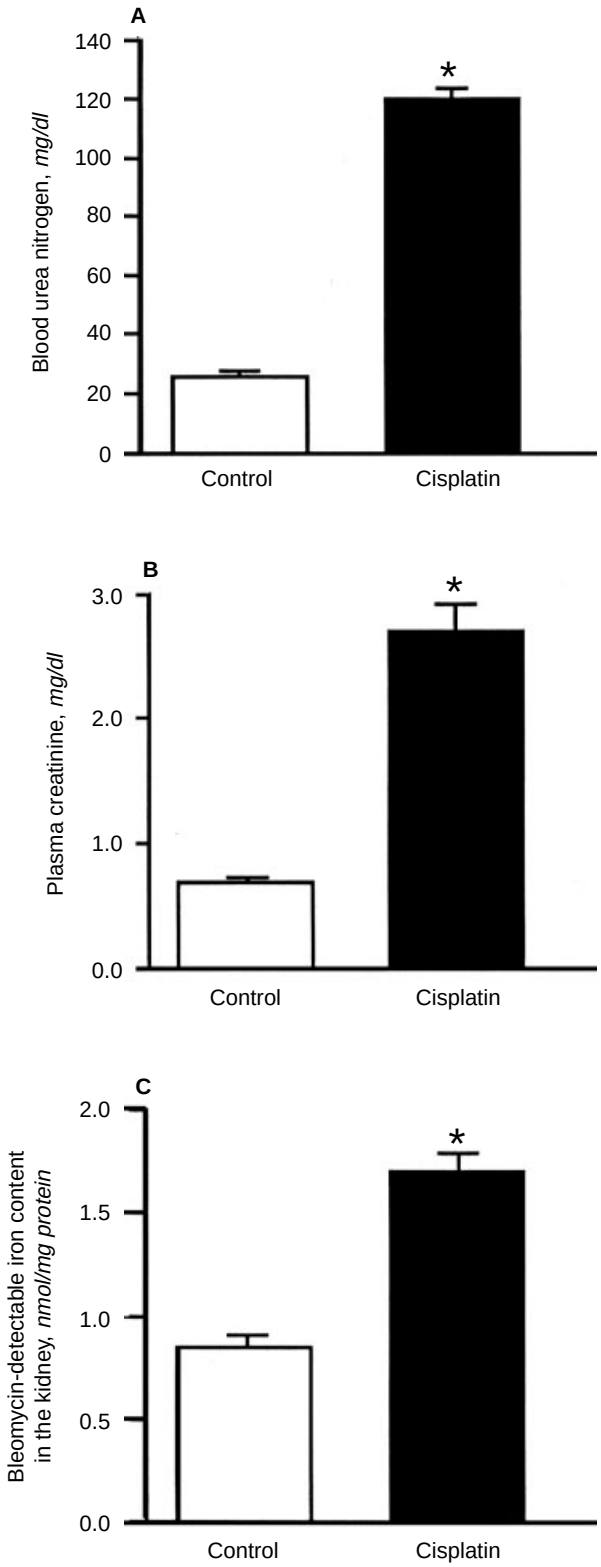

Fig. 4. Cisplatin-induced acute renal failure as measured by (*A*) blood urea nitrogen, (*B*) plasma creatinine and (*C*) bleomycin-detectable iron content in the kidney. Cisplatin model of acute renal failure in rats was induced by intraperitoneal injection of cisplatin at a dose of 10 mg/kg body wt. The animals were sacrificed 72 hours after cisplatin injection. Bleomycin-detectable iron was measured as described in the **Methods** section. Values are means ± SE, $N = 5$, $^*P < 0.01$ compared with control animals.

effect of DFO was unlikely to be due to chelation of cisplatin (data not shown).

Iron has been shown to participate in the generation of powerful oxidant species, such as the hydroxyl radical via the metal catalyzed Haber-Weiss reaction [5]. We thus examined the potential role of hydroxyl radical in cisplatin-induced cytotoxicity

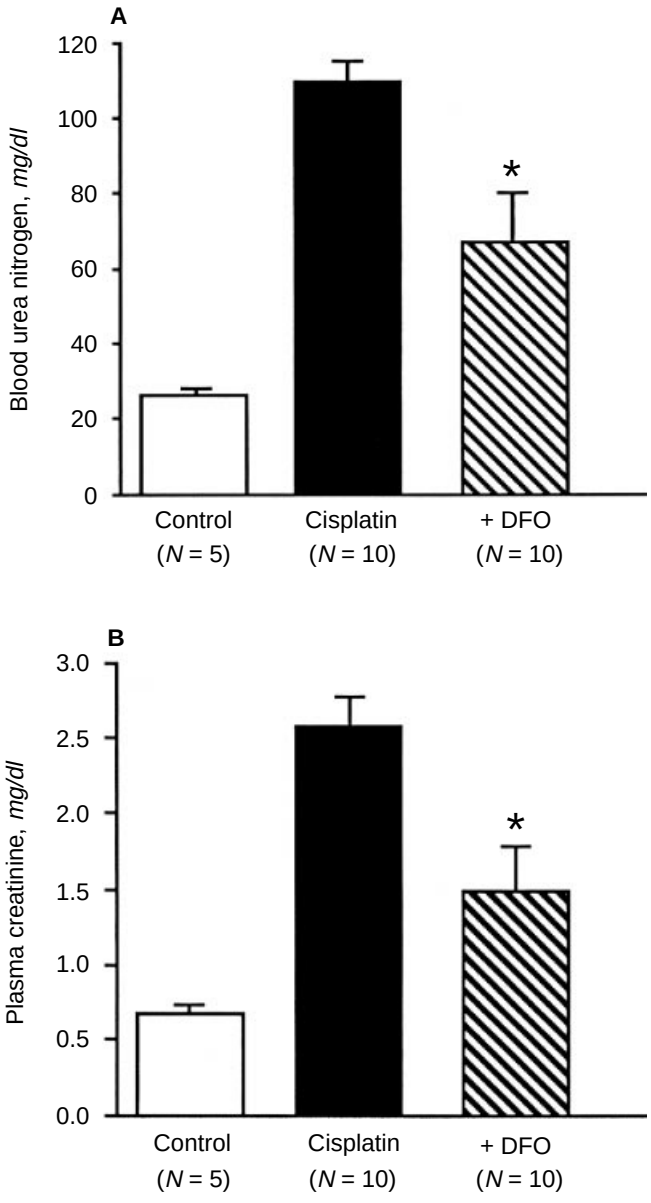

Fig. 5. Effect of iron chelator deferoxamine (DFO) on cisplatin-induced acute renal failure as measured by (*A*) blood urea nitrogen and (*B*) plasma creatinine. The cisplatin model of acute renal failure in rats was induced by intraperitoneal injection of cisplatin at a dose of 10 mg/kg body wt. DFO was administered via an osmotic pump which was implanted subcutaneously and delivered approximately 30 mg DFO per rat per day until sacrifice. The animals were sacrificed 72 hours after cisplatin injection. Values are means ± SE. The number of animals used is indicated. $^*P < 0.01$, compared with cisplatin treatment alone.

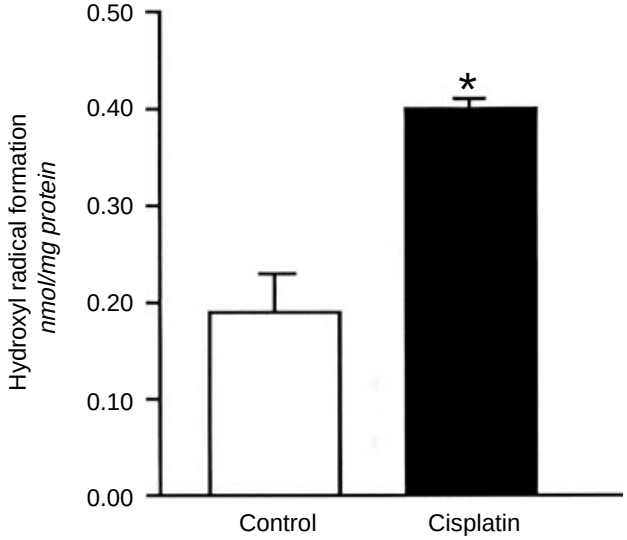

Fig. 6. Effect of cisplatin on hydroxyl radical formation in LLC-PK$_1$ cells. Confluent cell monolayer was incubated with 200 μg/ml of cisplatin for 2.5 hours in HBSS. 2-Deoxy-D-ribose was added to the medium just prior to the incubation. At the end of the incubation, the incubation medium was collected for the measurement of hydroxyl radical formation as described in the **Methods** section. Values are means ± SE, $N = 3$, $^*P < 0.01$, compared with control cells.

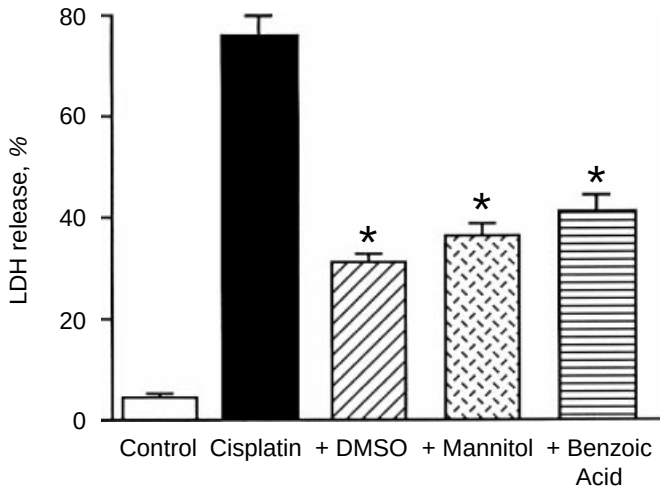

Fig. 7. Effect of hydroxyl radical scavengers on cisplatin-induced cytotoxicity as measured by lactate dehydrogenase (LDH) release in LLC-PK$_1$ cells. Confluent cell monolayer was incubated with 200 μg/ml of cisplatin for four hours. Hydroxyl radical scavengers including dimethyl sulfoxide (DMSO, 5 mM), mannitol (50 mM) and benzoic acid (100 mM) were added to the medium just prior to the addition of cisplatin. LDH release was measured at the end of incubation as described in the **Methods** section. Values are means ± SE. $N = 4$, $^*P < 0.01$, compared with cisplatin treatment alone.

both in *in vitro* and *in vivo* studies. As shown in Figure 6, there was a significant increase in the formation of hydroxyl radicals while exposing the LLC-PK$_1$ cells to cisplatin at a cytotoxic dose (200 μg/ml). We examined the effect of hydroxyl radical scavengers on cisplatin-induced cytotoxicity to LLC-PK$_1$ cells utilizing the doses similar to those that we have previously shown to scavenge hydroxyl radical in a cell-free system [20]. Compared to cells treated with cisplatin alone (LDH release, 76 ± 4%), hydroxyl radical scavengers, DMSO (31 ± 2%), mannitol (36 ± 2%), and benzoic acid (41 ± 3%, $N = 4$, $P < 0.01$) provided a marked protection against cisplatin-induced cytotoxicity in LLC-PK$_1$ cells (Fig. 7). In an *in vivo* study, rats treated concurrently with DMSO had significant protection (BUN, 49 ± 10 mg/dl; creatinine, 1.19 ± 0.20 mg/dl, $N = 7$, $P < 0.01$) compared to rats treated with cisplatin alone (BUN, 95 ± 10 mg/dl; creatinine, 2.00 ± 0.18 mg/dl, $N = 7$; Fig. 8 A, B). In a separate study, we examined the effect of another hydroxyl radical scavenger, DMTU and urea (which is not a hydroxyl radical scavenger but serves as a control)

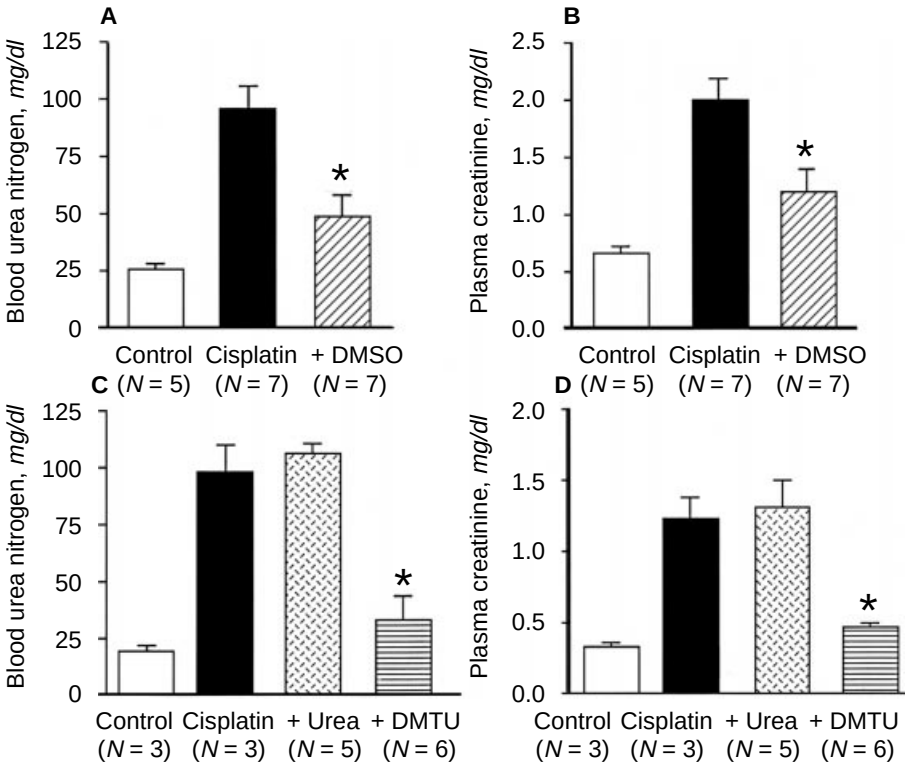

Fig. 8. Effect of hydroxyl radical scavengers dimethyl sulfoxide (DMSO) or dimethylthiourea (DMTU) on cisplatin-induced acute renal failure as measured by blood urea nitrogen (*A and C*) and plasma creatinine (*B and D*). Cisplatin model of acute renal failure in rats was induced by intraperitoneal injection of cisplatin at a dose of 10 mg/kg body wt. DMSO was administered via intraperitoneal injection twice a day at a dose of 4 g/kg body wt 4 hours prior to cisplatin injection until sacrifice. DMTU or urea (which is not a hydroxyl radical scavenger and serves as a control) was administered via intraperitoneal injection 30 minutes prior to cisplatin injection at a dose of 500 mg/kg body wt, followed by twice a day at a dose of 125 mg/kg body wt until sacrifice 72 hours after cisplatin injection. Values are means ± SE. The number of animals used is indicated. $^*P < 0.01$, compared with cisplatin treatment alone.

on cisplatin nephrotoxicity. As shown in Figure 8 C and D, rats pretreated with DMTU had significant protection (BUN, 33 ± 11 mg/dl; creatinine, 0.47 ± 0.03 mg/dl, $N = 6$, $P < 0.01$) compared to rats treated with cisplatin alone (BUN, 98 ± 12 mg/dl; creatinine, 1.23 ± 0.15 mg/dl, $N = 3$). However, the pretreatment of rats with urea had no effect on cisplatin nephrotoxicity (BUN, 106 ± 4 mg/dl; creatinine, 1.30 ± 0.20 mg/dl, $N = 5$). The lack of effect of urea indicates that the protective effect of DMSO or DMTU is most likely due to their ability to be oxidant scavengers rather than due to osmotic diuresis.

We also examined the histological changes in the kidney obtained from 5 rats in each of the groups (control, cisplatin alone, cisplatin + DFO, cisplatin + DMSO). Renal function studies in these subsets were not different from the overall experimental groups (control: BUN, 26 ± 2 mg/dl, creatinine, 0.69 ± 0.05 mg/dl; cisplatin alone: BUN, 121 ± 3 mg/dl, creatinine, 2.67 ± 0.25 mg/dl; cisplatin + DFO: BUN, 58 ± 20 mg/dl, creatinine, 1.37 ± 0.44 mg/dl; cisplatin + DMSO: BUN, 51 ± 11 mg/dl, creatinine, 1.20 ± 0.6 mg/dl). The histological changes were graded as described in the methods and the results expressed in Table 1. Compared to control rats (Fig. 9A), in rats treated with cisplatin there was extensive epithelial cell vacuolization, swelling, desquamation and necrosis (grade IV) occurring predominantly in the proximal convoluted tubules (Fig. 9B). Rats treated with the iron chelator, DFO (Fig. 9C), or the hydroxyl scavenger, DMSO (Fig. 9D), had a marked reduction in the extent of tubular damage (grade I-II).

Table 1. Semiquantitative analysis of renal tubule histology in rats

Group	*N*	Histologic grade				
		0-trace	I	II	III	IV
Control	5	5				
Cisplatin	5				3	2
+ Deferoxamine	5	1	2	1	1	
+ Dimethyl sulfoxide	5	2	2	1		

Five rats in each group were obtained for kidney histology study. Renal function study in these subsets was not different from the overall experimental groups. Renal tubule histologic grading is as follows: 0, normal; I, areas of tubular epithelial cell swelling, vacuolar degeneration, necrosis, and desquamation involving < 25% of cortical tubules; II, similar changes involving > 25% but < 50%; III, similar changes involving > 50% but < 75% of cortical tubules; IV, similar changes involving > 75%.

DISCUSSION

In the present study, we demonstrate that catalytic iron, as measured by bleomycin assay, was significantly increased in LLC-PK_1 cells exposed to cisplatin and in the kidney of rats treated with cisplatin. Iron chelators including DFO and 1,10-phenanthroline completely prevented cisplatin-induced cytotoxicity in LLC-PK_1 cells and DFO also provided marked protection against cisplatin-induced acute renal failure in rats. Taken together, these data suggest that the catalytic iron plays an important role in the pathogenesis of this model of renal injury.

An iron chelator, DFO, has been shown to substantially reduce the myocardial, hematological and hepatotoxicity induced by various chemotherapeutic agents [21, 22]. In previous studies [23], DFO has been shown to be effective in reducing the gastrointestinal side effects following the administration of cisplatin. In an *in vivo* study [24], pretreating with DFO in a single dose at 30 minutes prior to cisplatin administration was not protective against cisplatin nephrotoxicity despite a marked decrease in lipid peroxidation. The lack of protective effect may be related to the half-life of DFO when compared to the prolonged half-life of

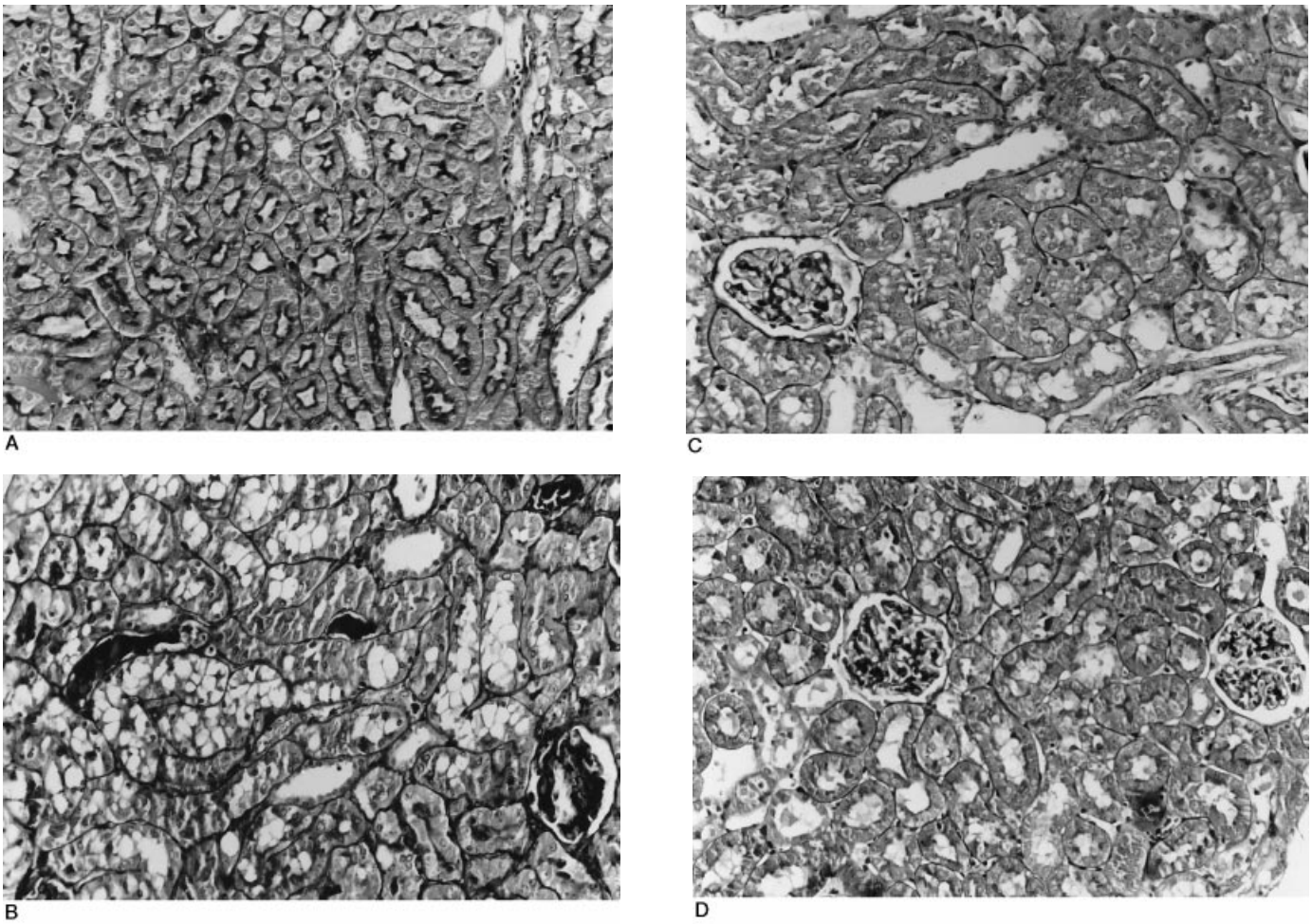

Fig. 9. Light-microscopy section of kidney. (*A*) Control rat showing no abnormalities. (*B*) Rat treated with cisplatin showing extensive epithelial cell vacuolization, swelling, desquamation and necrosis (grade IV) occurring predominantly in the proximal convoluted tubules. (*C*) Rat treated with the iron chelator, deferoxamine (DFO), and (*D*) the hydroxyl radical scavenger, dimethyl sulfoxide (DMSO), both of which show a marked reduction in the extent of tubular damage (grade I-II).

cisplatin in a seven-day model of renal injury, where there is substantial accumulation in the tissue that exceeds concentration in the plasma and other organs.

One of the important mechanisms by which iron mediates tissue injury is the generation of the highly toxic hydroxyl radical via the iron catalyzed Haber-Weiss reaction [5]. An increased hydroxyl radical formation has been demonstrated in renal tubular cells subjected to hypoxia/reoxygenation [12, 25]. Hydroxyl radical scavengers have also provided marked protection against ischemic [26], aminoglycoside-induced [9], as well as myoglobinuric acute renal failure [11]. The protective effects of iron chelators and hydroxyl radical scavengers in several models of renal injury have been generally taken as evidence for the participation of hydroxyl radical in tissue damage because iron is critical in the generation of hydroxyl radical via the Haber-Weiss reaction [9–11, 26]. Particularly relevant to the current study is the observation in which cisplatin has been shown to generate hydroxyl radical by interaction with DNA in a cell free system [27]. The present study demonstrated that exposure of LLC-PK_1 cells to cisplatin resulted in significant increase in hydroxyl radical formation. Hydroxyl radical scavengers, DMSO, mannitol and benzoic acid significantly reduced cisplatin-induced cytotoxicity and, treatment with DMSO or DMTU provided significant protection against cisplatin-induced acute renal failure.

We did not examine the mechanisms by which oxidants cause cisplatin cytotoxicity and acute renal failure. However, we and others have demonstrated an important role of iron in oxidant injury to renal tubular epithelial cells [13, 28], and demonstrated DNA fragmentation and endonuclease activation, both considered biochemical features of apoptosis, in oxidant-mediated injury to LLC-PK_1 cells [29]. In a recent study it was shown that cisplatin induces proximal tubular epithelial cell injury both by apoptosis with DNA fragmentation and chromatin condensation as well as necrosis in higher doses [30]. Thus, it is conceivable that cisplatin-induced cytotoxicity may be the result of oxidant-induced DNA damage. Based on cisplatin's ability to induce lipid peroxidation both *in vitro* and *in vivo*, lipid peroxidation is another iron-related mechanism that may be important in cisplatin cytotoxicity [31, 32]. In addition, treatment with antioxidants including α-tocopherol, N-N′-diphenyl-*p*-phenylendiamine and ascorbic acid, significantly attenuated cisplatin-induced acute renal failure in rats [33], or cytotoxicity in cultured renal epithelial cells [34].

In summary, our study clearly demonstrates that bleomycin-detectable iron is significantly increased in *in vitro* and *in vivo* models of cisplatin-induced nephrotoxicity. Treatment with both iron chelators and hydroxyl radical scavengers substantially prevented cytotoxicity and acute renal failure induced by cisplatin. Taken together, our data lend strong support to a critical role for iron in mediating tissue injury via hydroxyl radical formation in cisplatin-induced nephrotoxicity. However, additional studies would be necessary to determine whether iron chelators can prevent nephrotoxicity in humans as well as whether iron chelation therapy affects the tumorocidal effect of cisplatin.

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APPENDIX

Abbreviations used in this article are: BUN, blood urea nitrogen; DFO, deferoxamine; DMSO, dimethyl sulfoxide; DMTU, dimethylthiourea; HBSS, Hank's balanced salt solution; LDH, lactate dehydrogenase.

REFERENCES

1. Rozencweig M, von Hoff DD, Slavik M, Muggia FM: Cis-diamminedichloroplatinum (II). A new anticancer drug. *Ann Int Med* 86:803–812, 1977
2. Blachley JD, Hill JB: Renal and electrolyte disturbances associated with cisplatin. *Ann Int Med* 95:628–632, 1981
3. Ozols RF, Young RC: High-dose cisplatin therapy in ovarian cancer. *Semin Oncol* 12(Suppl 6):21–30, 1985
4. Safirstein R, Winston J, Moel D, Dikman S, Guttenplan J: Cisplatin nephrotoxicity: Insights into mechanism. *Int J Androl* 10: 325–346, 1987
5. Halliwell B, Gutteridge JMC: Role of free radicals and catalytic metal ions in human disease: An overview. *Meth Enzymol* 186:1–85, 1990
6. Gutteridge JMC, Rowley DA, Halliwell B, Westermarck T: Increased non-protein-bound iron and decreased protection against superoxide-radical damage in cerebrospinal fluid from patients with neuronal ceroid lipofuscinoses. *Lancet* 2:459–460, 1982
7. Baliga R, Ueda N, Shah SV: Increase in bleomycin-detectable iron in ischaemia/reperfusion injury to rat kidneys. *Biochem J* 291:901–905, 1993
8. Baliga R, Zhang Z, Baliga M, Shah SV: Evidence for cytochrome P-450 as a source of catalytic iron in myoglobinuric acute renal failure. *Kidney Int* 49:362–369, 1996
9. Walker PD, Shah SV: Evidence suggesting a role for hydroxyl radical in gentamicin-induced acute renal failure in rats. *J Clin Invest* 81:334–341, 1988
10. Paller MS, Hedlund BE: Role of iron in postischemic renal injury in the rat. *Kidney Int* 34:474–480, 1988
11. Shah SV, Walker PD: Evidence suggesting a role for hydroxyl radical in glycerol-induced acute renal failure. *Am J Physiol* 255:F438–F443, 1988
12. Paller MS, Neumann TV: Reactive oxygen species and rat renal epithelial cells during hypoxia and reoxygenation. *Kidney Int* 40:1041–1049, 1991
13. Walker PD, Shah SV: Hydrogen peroxide cytotoxicity in LLC-PK_1 cells: A role for iron. *Kidney Int* 40:891–898, 1991
14. Montine TJ, Borch RF: Quiescent LLC-PK_1 cells as a model for cis-diamminedichloroplatinum(II) nephrotoxicity and modulation by thiol rescue agents. *Cancer Res* 48:6017–6024, 1988
15. Baldew GS, Boymans AP, Mol JGJ, Vermeulen NPE: The influence of ebselen on the toxicity of cisplatin in LLC-PK_1 cells. *Biochem Pharmacol* 44:382–387, 1992
16. Halliwell B, Grootveld M, Gutteridge JMC: Methods for the measurement of hydroxyl radicals in biochemical systems: Deoxyribose degradation and aromatic hydroxylation. *Meth Biochem Anal* 33:59–90, 1988
17. Capasso G, Giordano DR, de Tommaso G, De Santo NG, Massry SG: Parathyroidectomy has a beneficial effect on experimental cisplatin nephrotoxicity. *Clin Nephrol* 33:184–191, 1990
18. Abul-Ezz SR, Walker PD, Shah SV: Role of glutathione in an animal model of myoglobinuric acute renal failure. *Proc Natl Acad Sci USA* 88:9833–9837, 1991
19. Zager RA: Combined mannitol and deferoxamine therapy for myohemoglobinuric renal injury and oxidant tubular stress. Mechanistic and therapeutic implications. *J Clin Invest* 90:711–719, 1992
20. Baliga R, Zhang Z, Shah SV: Role of cytochrome P-450 in hydrogen peroxide-induced cytotoxicity to LLC-PK_1 cells. *Kidney Int* 50:1118–1124, 1996
21. Al-Harbi MM, Al-Gharably NM, Al-Shabanah OA, Al-Bekairi AM, Osman AMM, Tawfik HN: Prevention of doxorubicin-induced myocardial and haematological toxicities in rats by the iron chelator desferrioxamine. *Cancer Chemother Pharmacol* 31:200–204, 1992
22. Al-Bekairi AM, Osman AMM, Hafeez MA, Al-Gharably NM, Al-Shabanah OA, Al-Harbi MM: Effect of desferrioxamine on the hepatoxicity of adriamycin in normal mice. *Drug Dev Res* 29:56–62, 1993
23. Matsuki N, Torii Y, Saito H: Effects of iron and deferoxamine on cisplatin-induced emesis: Further evidence for the role of free radicals. *Eur J Pharmacol* 248:329–331, 1993
24. Al-Harbi MM, Osman AMM, Al-Gharably NM, Al-Bekairi AM, Al-Shabanah OA, Sabah DM, Raza M: Effect of desferrioxamine on cisplatin-induced nephrototoxicity in normal rats. *Chemotherapy* 41:448–454, 1995
25. Paller MS, Jacob HS: Cytochrome P-450 mediates tissue-damaging hydroxyl radical formation during reoxygenation of the kidney. *Proc Natl Acad Sci USA* 91:7002–7006, 1994
26. Paller MS, Hoidal JR, Ferris TF: Oxygen free radicals in ischemic acute renal failure in the rat. *J Clin Invest* 74:1156–1164, 1984
27. Masuda H, Tanaka T, Takahama U: Cisplatin generates superoxide anion by interaction with DNA in a cell-free system. *Biochem Biophys Res Commun* 203:1175–1180, 1994
28. Andreoli SP, McAteer JA: Reactive oxygen molecule-mediated injury in endothelial and renal tubular epithelial cells in vitro. *Kidney Int* 38:785–794, 1990
29. Ueda N, Shah SV: Endonuclease-induced DNA damage and cell death in oxidant injury to renal tubular epithelial cells. *J Clin Invest* 90:2593–2597, 1992
30. Lieberthal W, Triaca V, Levine J: Mechanisms of death induced by cisplatin in proximal tubular epithelial cells: Apoptosis vs. necrosis. *Am J Physiol* 270:F700–F708, 1996
31. Hannemann J, Baumann K: Cisplatin-induced lipid peroxidation and decrease of gluconeogenesis in rat kidney cortex: Different effects of antioxidants and radical scavengers. *Toxicology* 51:119–132, 1988
32. Nakano S, Gemba M: Potentiation of cisplatin-induced lipid peroxidation in kidney cortical slices by glutathione depletion. *Jpn J Pharmacol* 50:87–92, 1989
33. Sugihara K, Gemba M: Modification of cisplatin toxicity by antioxidants. *Jpn J Pharmacol* 40:353–355, 1986
34. Gemba M, Fukuishi N: Amelioration by ascorbic acid of cisplatin-induced injury in cultured renal epithelial cells. *Contrib Nephrol* 95:138–142, 1991