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ORIGINAL ARTICLE

Hydroxyl radical scavenger ameliorates cisplatin-induced nephrotoxicity by preventing oxidative stress, redox state unbalance, impairment of energetic metabolism and apoptosis in rat kidney mitochondria

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Abstract Nephrotoxicity is the major dose-limiting factor of cisplatin chemotherapy. Reactive oxygen species generated in mitochondria are thought to be the main cause of cellular damage in such injury. The present study examined, in vivo, the protective potential of the hydroxyl radical scavenger dimethylthiourea (DMTU) against cisplatin-induced effects on renal mitochondrial bioenergetics, redox state and oxidative stress. Adult male Wistar rats (200 to 220 g) were divided into four groups of eight animals each. The control group was treated only with an intraperitoneal (i.p.) injection of saline solution (1 ml/100 g body weight). The second group was given only DMTU (500 mg/kg body weight, i.p, followed by 125 mg/Kg, i.p., twice a day until they were killed). The third group was given a single injection of cisplatin (10 mg/kg body weight, i.p.). The fourth group was given DMTU (500 mg/kg body weight, i.p.), just before the cisplatin injection (10 mg/kg body weight, i.p.), followed by injections of DMTU (125 mg/kg body weight, i.p.) twice a day until they were killed. Animals were killed 72 h after the treatment. Besides not presenting any direct effect on mitochondria, DMTU substantially inhibited cisplatin-induced mitochondrial injury and cellular death by apoptosis, suppressing the occurrence of acute renal failure. All the following cisplatin-induced effects were prevented by DMTU: (1) increased plasmatic levels of creatinine and blood urea nitrogen (BUN); (2) decreased ATP content, calcium uptake and electrochemical potential; (3) oxidation of lipids, including cardiolipin; and oxidation of proteins, including sulfhydryl, and aconitase enzyme, as well as accumulation of carbonyl proteins; (4) depletion of the antioxidant defense (NADPH and GSH) and (5) increased activity of the apoptosis executioner caspase-3. Our findings show the important role played by mitochondria and hydroxyl radicals in cisplatin-induced nephrotoxicity, as well as the effectiveness of DMTU in preventing the renal mitochondrial damage caused by cisplatin. These results strongly suggest that protection of mitochondria by hydroxyl radical scavengers may be an interesting approach to prevent the kidney tissue damage caused by cisplatin-chemotherapy.

Keywords Cisplatin · DMTU · Mitochondria · Nephrotoxicity · ROS · Cytoprotection · Antioxidant

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Introduction

Cisplatin (*cis*-diamminedichloroplatinum II) is an effective chemotherapeutic agent used in the treatment of a wide range of tumors. Despite its effectiveness as an anti-tumor drug, nephrotoxic side effects have significantly restricted its clinical use [1]. In spite of the intensive prophylactic measures used, such as aggressive hydration and forced diuresis, irreversible renal damage occurs in about one-third of cisplatin-treated patients [2, 3]. Several lines of evidence suggest that mitochondrial damage and reactive oxygen species (ROS) are involved in the nephrotoxicity of cisplatin [4, 5]. Although mitochondria contain an antioxi-

dant defense system, they also appear to be the main intracellular source of ROS, such as superoxide anions and hydroxyl radicals [6, 7]. Recently, strategies are being developed for the targeted delivery of antioxidants to mitochondria with the purpose of reducing toxic oxidative stress injuries [2, 8, 9]. Therefore, the selective protection of mitochondria to counteract or at least minimize cisplatin-induced toxicity against healthy tissues, such as kidney, is of great importance in the chemotherapy of patients with cancer.

The exact roles of free radicals in cisplatin-induced nephrotoxicity as well as the mechanisms responsible for the beneficial effects of free radical scavengers have not been completely evaluated. There is a concern that antioxidants might interfere with the anticancer activity. Some studies demonstrate the reduction in the efficacy of chemotherapy or radiation when combined with antioxidants. However, the vast majority of both in vivo and in vitro studies suggest that certain antioxidants, given under cautious and judicious conditions, can significantly reduce the toxicity of chemotherapeutic agents and, instead of interfering with their antitumor effect, can actually enhance it or have no effect on their action. [6, 10–13]. Most of the compounds that ameliorate cisplatin-induced nephrotoxicity contain a nuclephilic sulphur atom, which might bond to the platinum ion, neutralizing its cytotoxic effects without impairing its effectiveness [4].

Dimethylthiourea (DMTU) is a hydroxyl radical scavenger whose beneficial effect against cytotoxicity has been described in different tissues and pathologies, but nevertheless, the exact mechanisms by which this action is exerted, especially those involving mitochondria, have not been fully elucidated.

The DMTU has been demonstrated to be highly effective in minimizing diabetic neurovascular complications, which are associated to increased oxidative stress [14]. According to Roychoudhury et al. [15] pretreatment with DMTU prevented hydroxyl radical formation and reduced the lipid peroxidation induced by H_2O_2 in mitochondria of pulmonary vascular smooth muscle tissue. DMTU has been shown to prevent cisplatin-induced acute renal failure in a mechanism associated with a decrease in apoptosis through mitochondrial pathways [16] and through death receptors pathways [13]. In a previous study, Baliga et al. [2] demonstrated, in vitro and in vivo, the protective effect of DMTU against cisplatin-induced renal failure, without examining the mechanisms by which it occurs.

In the present study we examined, in vivo, the molecular events underlying the antioxidant protection against the nephrotoxicity induced by cisplatin. For this purpose we evaluated the mechanisms involved in the protective effect of DMTU against mitochondrial dysfunction, oxidative stress, apoptotic cell death and redox state unbalance induced by cisplatin in rat kidney mitochondria. The understanding of these mechanisms could greatly contribute to the improvement of the renal cytoprotection approach during the chemotherapy with cisplatin. Since mitochondrial damage constitutes an initial stage in the cisplatin-induced nephrotoxicity, targeting antioxidants to mitochondria is a promising new field that may enhance chemotherapy with this important anticancer agent.

Materials and methods

Chemicals

cis-Diammineplatinum (II) dichloride (cisplatin), dimethylthiourea (DMTU), rhodamine 123, rotenone, ADP, ATP and NADPH were obtained from Sigma-Chemical Co. (St. Louis, MO, USA). Other reagents were analytical grade. Heparin (liquemine®) was obtained from Roche (Rio de Janeiro, RJ, Brazil). Sodium thiopental was obtained from Cristalia (Itapira, SP, Brazil). All solutions were prepared with ultra-pure water purified by a Milli-Q Gradient system (Millipore, Bedford, USA). Cisplatin solution was prepared in saline.

Animals

Research protocols were in strict accordance with the "Ethical principles and guidelines for experiments on animals" of the Swiss Academy of Medical Sciences and Swiss Academy of Sciences.

Animals treatment

Male Wistar rats (200–220 g) were housed four per cage and were maintained in a 12-h light/dark cycle in a temperature and humidity controlled facility. Standard rat chow and water were provided *ad libitum*. Animals were divided into four groups, with eight rats in each group. The control group (vehicle) received a single intraperitoneal (i.p.) injection of saline solution (1 ml/100 g body weight). The second group (DMTU) was given an injection of DMTU (500 mg/kg body weight, i.p) followed by intraperitoneal injections of 125 mg/kg twice a day until they were killed. The third group (DMTU + Cisplatin) received DMTU (500 mg/kg body weight, i.p.) just before cisplatin injection (10 mg/kg body weight, i.p.) followed by intraperitoneal injections of DMTU (125 mg/kg body weight) twice a day until they were killed. The fourth group (cisplatin, $n = 8$) was given a single intraperitoneal injection of cisplatin (10 mg/kg body weight). All animals, previously anaesthetized with an intraperitoneal injection of 60 mg sodium pentobarbitone/kg body weight

[17] were killed by cervical dislocation after 72 h. Blood samples were collected using heparin as anticoagulant for the measurement of blood urea nitrogen (BUN) and plasma creatinine.

Preparation of mitochondrial fraction

Rats were killed, and the kidneys were immediately removed and washed in cold saline solution. Medullae was removed and the remaining cortex was cut into pieces, and then minced and homogenized with a Potter-Elvehjem (three times for 15 s, 1-min intervals) in a medium containing 250 mM sucrose, 1 mM EGTA, 0.2% bovine serum albumin and 10 mM Hepes-KOH, pH 7.4. Kidney mitochondria were isolated by standard differential centrifugation [18, 19]. The homogenate was centrifuged at 755*g* for 5 min, and the resulting supernatant was centrifuged at 13,300*g* for 10 min. The pellet was resuspended in 10 ml of medium containing 250 mM sucrose, 0.1 mM EGTA, 0.14% bovine serum albumin and 10 mM Hepes-KOH, pH 7.4, and centrifuged at 13,300*g* for 10 min. All procedures of centrifugation were performed at 4°C. The mitochondrial pellet obtained was resuspended in 1 ml medium containing 160 mM KCl and 10 mM Hepes-KOH, pH 7.4. Mitochondrial suspension was kept on ice and used within 3 h. Protein concentration was determined by the biuret method with bovine serum albumin as a standard.

Mitochondrial membrane potential

The electrical potential of the mitochondrial inner membrane was assessed by monitoring the mitochondrial uptake of rhodamine 123 in a fluorescence spectrophotometer (F-2500, Hitachi) operating at an emission wavelength of 535 nm and an excitation wavelength of 505 nm. One milligram of mitochondrial protein was used and the incubation medium had the following composition: 160 mM KCl, 8.5 mM potassium phosphate, 10 mM Hepes-KOH, pH 7.4, added of 5 μM rotenone and 5 μM rhodamine 123. The respiratory substrate was 10 mM succinate. The reaction was initiated with 0.4 μmol ADP and the assay was performed at 30°C [20, 21].

Ca^{2+} influx measurement

The kinetics of Ca^{2+} uptake by mitochondria was studied by a spectrophotometric method with Arsenazo III, a free calcium indicator, at wavelengths of 685 and 675 nm. The following incubation medium (2 ml final volume) was used: 160 mM KCl, 10 mM Hepes-KOH, 5 μM rotenone, 20 μM CaCl and 25 μM arsenazo III, pH 7.4. One milligram of mitochondrial protein was used. The assay was performed at 30°C and calcium uptake was induced by 10 mM potassium succinate [22].

ATP synthesis

A portion of 100 mg of kidney was homogenized and extracted with 1 M $HClO_4$ and centrifuged at 14,000*g* for 5 min. The resulting supernatant was then neutralized with 2 M KOH/100 mM Tris–HCl (pH 7.8) and centrifuged at 14,000*g* for 10 min. ATP content was measured in the supernatant by a Sigma ATP Bioluminescent Assay Kit based on the luciferin–luciferase method, and it was performed in an EG&G BERTHOLD chemiluminescence analyzer, model AutoLumat LB 953 [23, 24].

Lipid peroxidation

Malonaldehyde (MDA) and 4-hydroxy-2(E)-nonenal (4-HNE) were determined using the assay kit "Lipid Peroxidation Assay" (Cat. no 437634, Calbiochem). Absorbance of the stable chromophore yielded was measured in a Hitachi spectrophotometer (model U-3000) at 586 nm. One milligram of mitochondrial protein was used.

Assay of mitochondrial cardiolipin content

Cardiolipin content of mitochondria was measured using the cardiolipin specific dye 10-*N*-nonyl-acridine orange (NAO). One milligram of mitochondrial protein was incubated at 30°C for 45 min in the presence of NAO 5 μM, in the medium containing 160 mM KCl and 10 mM Hepes-KOH, pH 7.4. The excess dye was washed out by centrifugation, and the mitochondrial pellet appropriately diluted in the same buffer. Fluorescence was determined in a Hitachi spectrofluorimeter (model F-2500) operating at 485 nm (ex) and 535 nm (em). The fluorescence was converted to relative fluorescence units using quinine (1 mg/ml in 0.1N H_2SO_4, λex 360 nm, λem 457 nm) as the reference [25,26].

Sulfhydryl protein (P-SH)

The content of P-SH was evaluated as previously described by Grattagliano et al. [27]. Proteins of the mitochondrial suspension (4 mg) were precipitated with sulphosalicilic acid 4% and centrifuged at 4,500*g* for 5 min. The pellet was washed twice with 2% sulphosalicilic acid to remove free thiols and resuspended in 200 μl of 6 M Guanidine pH 6.0. The final samples were read spectrophotometrically at 412 and 530 nm. Next, 50 μl of 10 mM 5,5-dithiobis (2-nitrobenzoic acid) was added and after 30 min of incubation in the dark, samples were read again at 412 and 530 nm. P-SH concentrations were calculated using a standard curve prepared with reduced GSH.

Carbonyl proteins

The measurement of carbonyl proteins was performed according to the method of Sedlak and Lindsay [28]. Mitochondrial suspension (2 mg) was treated with 1 ml of 0.2% dinitrophenylhydrazine (DNPH) in 2 N HCl or with 1 ml of 2 N HCl (control blank). Samples were incubated for 1 h at room temperature and stirred at 5-min intervals. Next, 200 µl of 50% TCA were added and the precipitated proteins were washed 3 times with 1:1 ethanol/ethylacetate and 3 times with 10% TCA. The final precipitate was dissolved in 2 ml of 6 M guanidine. The different spectrum of the DNPH-derivatives versus HCl controls was followed spectrophotometrically at 345–370 nm. The concentration of carbonyl groups was calculated from the spectrum absorbance, using 21.5 mM^{-1} cm^{-1} as the extinction coefficient for aliphatic hydrazones.

Aconitase activity assay

The activity of aconitase was assayed by determining the rate of formation of the intermediate product, *cis*-aconitate, from the substrate-citrate in the interconversion of L-citrate and isocitrate. The enzymatic reaction was initiated by adding 30 µl of the mitochondrial suspension in a total of 1 ml assay buffer containing 20 mM triethanolamine–HCl (pH 7.5) and 1.0 mM L-citrate. The changes of absorbance at 240 nm were recorded for 5 min by a MultiSpec 1501 Shimadzu spectrophotometer and used for the calculation of the enzyme activity. Aconitase activity was expressed as the formation of nmol *cis*-aconitate/mg protein/min [29].

Pyridine nucleotide (NADPH) oxidation

The oxidation of pyridine nucleotides was measured spectrofluorimetrically at 450 nm following excitation at 340 nm as previously described by Lund et al. [30]. One milligram of mitochondrial protein per ml of medium (250 mM sucrose and 10 mM Tris–HCl, pH 7.4) was used.

Glutathione assay

Oxidized and reduced glutathione were measured by the enzyme recycling method of Tietze [31] with some modifications. Mitochondrial suspension (1 mg protein) was added of 500 µl of 0.4 M $HClO_4$ and centrifuged at 10,000*g* for 10 min at 4°C. To determine GSH, a 50 µl aliquot of the supernatant was combined in a cuvette with 500 µl of buffer (1 mM EDTA, 3 mM NADPH, 100 mM potassium phosphate, pH 7.4). Next, 50 µl of 10 mM DTNB (5,5-dithiobis 2-nitrobenzoic acid) was added. After incubating for 1 min, 100 µl of glutathione reductase (5 U/ml) was added, and absorbance at 412 nm was monitored for 5 min. GSSG was measured in the samples previously neutralized with 100 mg of sodium carbonate and treated with 4 µl of 2-vinylpyridine. After 1 h of incubation in the dark, samples were centrifuged at 3,000*g* for 5 min and the supernatant was assayed as described above for GSH determination. A calibration curve submitted to the same procedure was used as reference to calculate GSH and GSSG concentrations.

Caspase 3 activity

Kidney homogenate was centrifuged at 10,000*g* for 10 min, and the resulting supernatant was diluted to 2 mg/ml in the homogenizing medium as previously described. A 50 µl aliquot of supernatant was assayed using the commercial kit "Caspase-3 Assay kit, Fluorimetric®" (Sigma).

Renal function

Plasma BUN and creatinin levels were measured using commercially available diagnostic kits (Labtest®).

Statistical analyses. All data were expressed as mean ± SD. Statistical analyses were conducted by Student's *t* test and were performed using the GraphPad Prism, version 3.0 for Windows (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was chosen as the level of significance.

Results

The following criteria were adopted to determine the significance levels: (1) the cisplatin-treated group was compared to the control group, which was treated only with saline solution, to validate the model of acute renal failure; (b) the DMTU + cisplatin-treated group was compared to the cisplatin-treated group in order to investigate the possible protective effect; (c) finally, the DMTU-treated group, to which cisplatin was not given, was compared to the control group in order to investigate the possibility of any direct effect of DMTU on mitochondria besides the protective effect against cisplatin-induced nephrotoxicity. Since no significant differences were observed between these two groups, the occurrence of any effect of DMTU on mitochondria, which could intervene in the validity of the experimental model, was excluded.

Cisplatin-induced acute renal failure

Cisplatin-induced nephrotoxicity is associated with the increase in plasma levels of creatinine and blood urea nitrogen (BUN), and is reported to be maximal at 3–5 days after cisplatin injection to the experimental animals [32, 33].

In the present study, plasma BUN and creatinine levels were significantly increased ($P < 0.05$) in the cisplatin group as compared to the saline control group (from 80.2 and 0.54 to 232.5 and 1.95 mg/100 ml, respectively), confirming the occurrence of cisplatin-induced renal failure, thereby validating the experimental model.

Effect of DMTU on cisplatin model of acute renal failure

In the DMTU + cisplatin-treated group, the average BUN level was 93.58 (±15.52) mg/100 ml and the average plasma creatinine level was 1.08 (±0.8) mg/100 ml, showing a significant protective effect of DMTU against acute renal failure ($P < 0.05$, in both cases). BUN and plasma creatinine results are represented in Fig. 1.

Effect of DMTU on renal mitochondrial function

ATP synthesis was assessed by the measurement of kidney ATP content (Fig. 2), which was significantly decreased in the cisplatin-treated group (75% of control). DMTU was able to prevent the decrease in ATP content, whose level was maintained practically unchanged (96.71% of control).

Cisplatin caused a substantial decrease in mitochondrial calcium uptake, evaluated through arsenazo III absorbance decay rate, which was significantly faster in the control group. In the DMTU + cisplatin group, the trace decay was very different from that of the cisplatin group, showing a profile closer to that obtained in the control group. After a 30-min run, arsenazo III absorbance was approximately 10-fold higher in the cisplatin-treated group (0.059) as compared to the control group (0.005). In the DMTU + cisplatin group, after a 30-min run, arsenazo III absorbance was

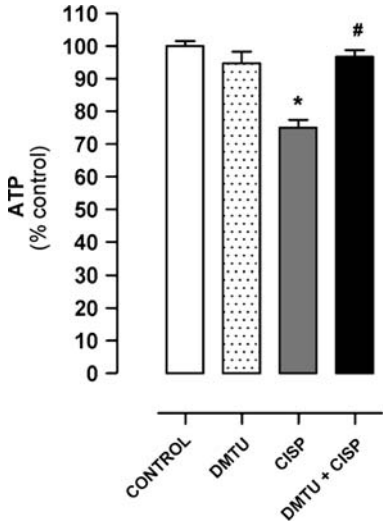

Fig. 2 Effect of cisplatin and DMTU on ATP synthesis. ATP content of the control group was considered 100%. Data expressed as mean ± SEM ($n = 8$). Analytical and experimental conditions were described in Materials and methods. * Significantly different from control group ($P < 0.05$). # Significantly different from cisplatin group ($P < 0.05$)

significantly different (0.012) from that of the cisplatin group, indicating a considerable protection. Traces of the kinetic analysis are represented in Fig. 3.

The mitochondrial membrane potential assay is presented in Fig. 4. With the addition of an oxidizable substrate (potassium succinate), a similar transmembrane electrical potential, assessed by rhodamine 123 fluorescence decay rate, was developed by all groups. The addition of ADP, which induced the metabolic transition to state 3, caused a decrease in the mitochondrial membrane

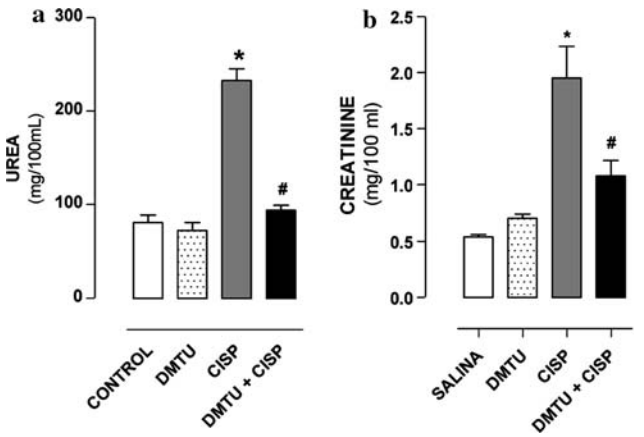

Fig. 1 Effect of cisplatin and DMTU on renal function of rats. Plasma levels of urea (**a**) and creatinine (**b**). Data were expressed as mean ± SEM ($n = 8$). Analytical and experimental conditions were described in Materials and methods. * Significantly different from control group ($P < 0.05$) # Significantly different from cisplatin group ($P < 0.05$)

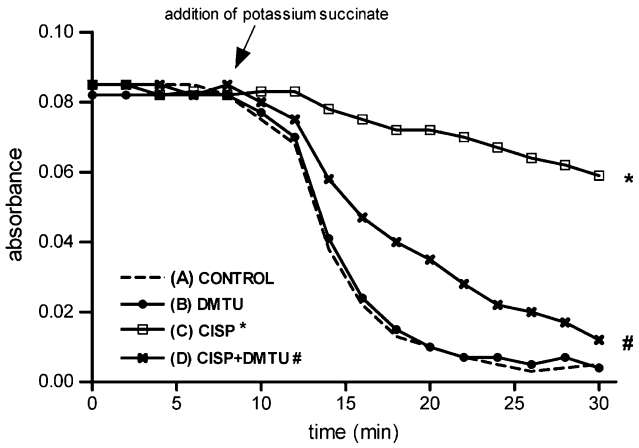

Fig. 3 Effect of cisplatin and DMTU on mitochondrial calcium uptake. The *traces* shown are representative of different experiments ($n = 8$). Analytical and experimental conditions were described in Materials and methods. * Significantly different from control group ($t = 30$ min, $P < 0.05$). # Significantly different from cisplatin group ($t = 30$ min, $P < 0.05$)

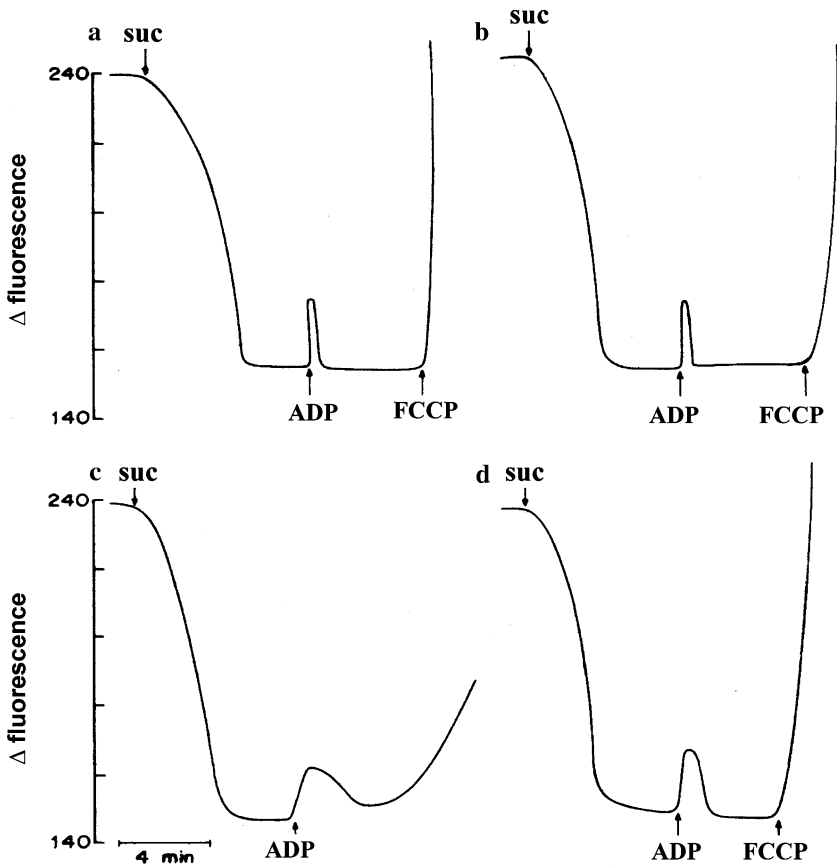

Fig. 4 Effect of cisplatin and DMTU on inner mitochondrial membrane electrochemical potential. **a** control group; **b** DMTU group; **c** cisplatin group; **d** DMTU + cisplatin group. The *traces* shown are representative of different experiments ($n = 8$). Analytical and experimental conditions were described in Materials and methods. *FCCP* carbonylcyanide-p-trifluoromethoxyphenylhydrazone; *suc* potassium succinate

potential, which corresponded to the energy used for ATP synthesis. When the phosphorylation cycle was completed, membrane potential quickly resumed to the previous level in the control group (trace a). By contrast, mitochondria from the cisplatin-treated group took longer to phosphorylate exogenous ADP. Furthermore, the mitochondrial electrochemical potential did not resume to the pre-ADP level, but decreased progressively (trace c). In the DMTU + cisplatin-treated group (trace d), even though ADP phosphorylation was slower than that of control, the initial mitochondrial electrochemical potential was developed again. The addition of the known uncoupler FCCP immediately collapsed the transmembrane electrical potential. No significative differences were observed between the control group (trace a) and the DMTU group (trace b).

Effect of DMTU on oxidative stress and on renal mitochondrial redox state

The concentrations of lipid peroxidation secondary products, MDA (malondialdehyde) and 4-HNE (4-hydroxy-2 (E) nonenal), were 9.87 nmol/mg protein in the control group and 15.72 nmol/mg protein in the cisplatin group, indicating the induction of lipid peroxidation by cisplatin. Accordingly, the content of the mitochondrial lipid cardiolipin was decreased in the cisplatin group (73% of control), probably due to oxidation processes. In the DMTU + cisplatin group, protection against lipid peroxidation was evidenced by low levels of MDA and 4-HNE (8.73 ± 1.56 nmol/mg protein, $P < 0.05$). Levels of cardiolipin in the DMTU + cisplatin group were significantly different from those of cisplatin group and very close to those of control ($96.59\% \pm 6.47$). Data related to lipid peroxidation and cardiolipin content are presented in Figs. 5 and 6, respectively.

An accumulation of oxidized proteins (Fig. 7), assessed by carbonyl and sulfhydryl proteins determination, was also observed in the mitochondria of cisplatin-treated rats. Compared to the control group, the cisplatin group showed a significant increase in carbonylated protein content (from 0.53 to 1.24 nmol/mg protein) and a significant decrease in sulfhydryl protein concentration (from 68.04 to 45.00 nmol/mg protein). Levels of sulfhydryl protein and carbonylated protein were, respectively, 76.37 ± 11.94 nmol/mg protein and 0.63 ± 0.04 nmol/mg protein in the DMTU + cisplatin group, indicating the occurrence of protection in this group. Furthermore, the activity of the mitochondrial protein aconitase, which was decreased in the cisplatin group (80.91% of control), was kept unaltered in the DMTU + cisplatin group (108.20% of control). Data are presented in Fig. 8.

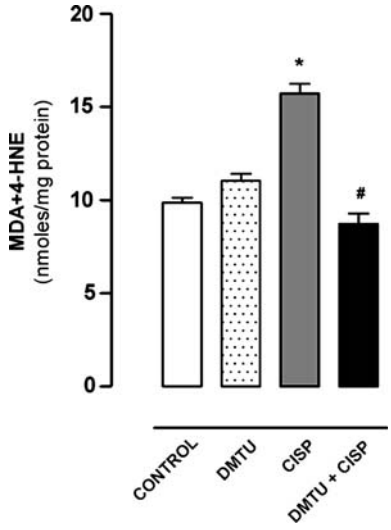

Fig. 5 Effect of cisplatin and DMTU on lipid peroxidation, assessed by MDA and 4-HNE levels. Data were expressed as mean ± SEM ($n = 8$). Analytical and experimental conditions were described in Materials and methods. * Significantly different from control group ($P < 0.05$). # Significantly different from cisplatin group ($P < 0.05$)

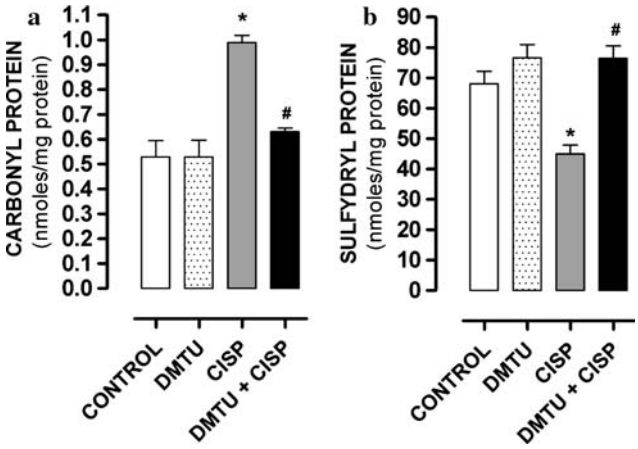

Fig. 7 Effect of cisplatin and DMTU on mitochondrial proteins, assessed by **a** carbonyl protein formation and **b** sulfhydryl protein levels. Data were expressed as mean ± SEM ($n = 8$). Analytical and experimental conditions were described in Materials and methods. * Significantly different from control group ($P < 0.05$). # Significantly different from cisplatin group ($P < 0.05$)

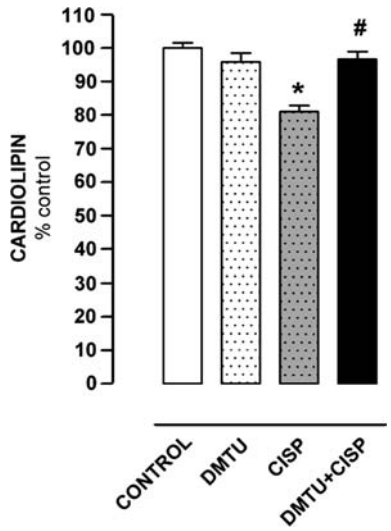

Fig. 6 Effect of cisplatin and DMTU on mitochondrial lipid cardiolipin. Data were expressed as mean ± SEM ($n = 8$). Analytical and experimental conditions were described in Materials and methods. * Significantly different from control group ($P < 0.05$). # Significantly different from cisplatin group ($P < 0.05$)

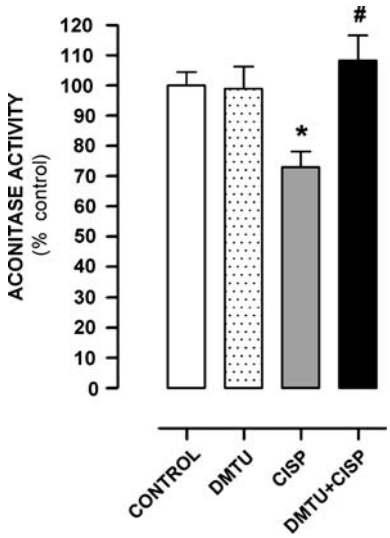

Fig. 8 Effect of cisplatin and DMTU on mitochondrial aconitase activity. Data were expressed as mean ± SEM ($n = 8$). Analytical and experimental conditions were described in Materials and methods. * Significantly different from control group ($P < 0.05$). # Significantly different from cisplatin group ($P < 0.05$)

Levels of GSH and GSH/GSSG ratio in the cisplatin-treated group were 2.38 ± 0.35 nmol/mg protein and 37.77 ± 5.48, respectively, which were significantly lower than those found in the control group (3.69 ± 0.35 nmol/mg protein and 90.24 ± 8.63, respectively), whereas GSSG content was significantly higher in the cisplatin-treated group (0.060 ± 0.003 nmol/mg protein) compared to the control group (0.041 ± 0.004 nmol/mg protein). No alterations in the GSH and GSSG levels were found in the DMTU + cisplatin group (3.65 ± 0.61 and 0.047 ± 0.007 nmol/mg protein, respectively) as compared to the control group. Therefore, the mitochondrial redox state, given by the GSH/GSSG ratio, which was significantly altered in the cisplatin group (37.77 ± 5.48) as compared to control (90.24 ± 8.63), was significantly higher in the DMTU + cisplatin group (77.02 ± 9.37), clearly indicating the beneficial effect of DMTU. Content of the pyridine nucleotide NADPH was significantly decreased in the

cisplatin-treated group (74.37% of control), but remained unchanged in the DMTU + cisplatin group (102.91% of control). The protective effect of DMTU on mitochondrial antioxidant defense system is presented in Fig. 9.

Cisplatin-related cell death by apoptosis

The occurrence of cell death by apoptosis in the kidney of cisplatin-treated rats, assessed by caspase-3 activity, was increased (184%) as compared to the control group. Protection was clearly evidenced by the unchanged enzyme activity in the DMTU + cisplatin group (108.81% of control). Data are presented in Fig. 10.

Discussion

Although reactive oxygen species (ROS) are regarded as a central key in cisplatin-induced nephrotoxicity, the exact roles of free radicals and the mechanisms involved in the protective effect of antioxidants have not been entirely explained [34].

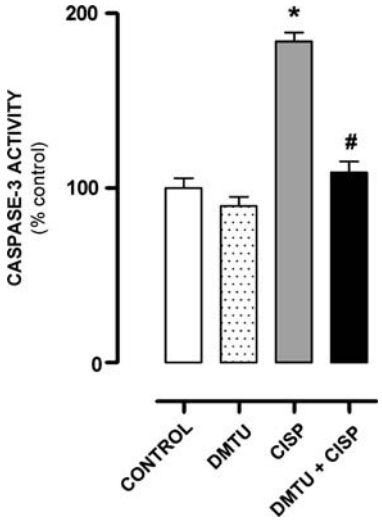

Fig. 10 Effect of cisplatin and DMTU on the executioner caspase-3 activity. Data were expressed as mean ± SEM ($n = 8$). Analytical and experimental conditions were described in Materials and methods. * Significantly different from control group ($P < 0.05$). # Significantly different from cisplatin group ($P < 0.05$)

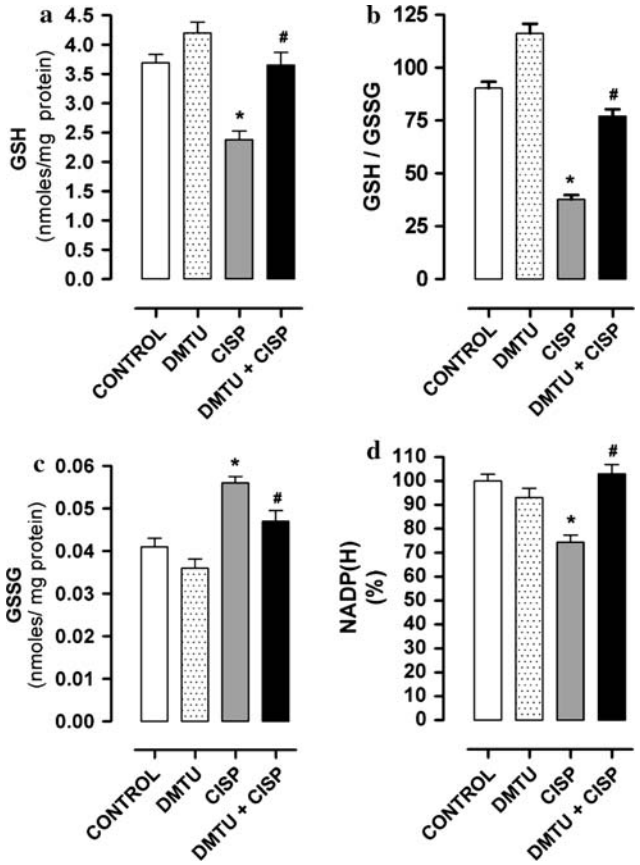

Fig. 9 Effect of cisplatin and DMTU on mitochondrial antioxidant defense system, assessed by **a** GSH levels, **b** GSSG levels, **c** GSH/GSSG ratio and **d** NADPH levels. Data were expressed as mean ± SEM ($n = 8$). Analytical and experimental conditions were described in Materials and methods. * Significantly different from control group ($P < 0.05$). # Significantly different from cisplatin group ($P < 0.05$)

DMTU is a powerful scavenger of hydroxyl radicals, and it has been used to test the involvement of hydroxyl radicals in several animal models of human disease. Furthermore, its beneficial effects in the prevention of cytotoxicity have been described in different tissues. Cameron et al. [14] reported the high effectiveness of DMTU in the treatment of diabetic neuropathy and vascular disease, which have been linked to increased oxidative stress. Baliga et al. [2] presented evidence of the critical role of iron in mediating tissue damage via hydroxyl radical formation in cisplatin-induced nephrotoxicity. Roychoudhury et al. [15] reported that the pretreatment of bovine pulmonary artery smooth muscle tissue with DMTU prevented hydroxyl radical production and markedly reduced lipid peroxidation induced by H_2O_2, without altering iron release. In our study we showed the effectiveness of the hydroxyl radical scavenger, DMTU, in the prevention not only of lipid peroxidation, assessed by MDA and 4-HNE levels, but also in the prevention of many alterations underlying the renal mitochondrial damage induced by cisplatin, such as decreased ATP synthesis, calcium uptake, electrochemical potential, cardiolipin content, sulfhydryl protein content, aconitase enzyme activity and GSH levels, as well as increased carbonylated protein and GSSG levels.

The DMTU has been shown to ameliorate cisplatin-induced nephrotoxicity by playing a role in different processes underlying this event. Gemba et al. [35] demonstrated that DMTU inhibited the increased urinary excretion of the enzymes glutamyltranspeptidase (gamma-GTP) and

N-acetyl-beta-D-glucosaminidase (NAG) caused by cisplatin together with GSH depletors. Baek et al. [16] showed that DMTU did not prevent cisplatin-induced necrotic cell death, but inhibited cisplatin-induced apoptotic cell death via inhibition of cytochrome c release and caspase activation (mitochondrial pathway). Based on these data they suggested that hydroxyl radicals are not involved in the process of necrotic cell death, but associated with cisplatin-induced apoptotic cell death. On the other hand, Tsuruya et al. [13] showed that DMTU ameliorated cisplatin-induced acute renal failure through inactivation of death receptor-mediated apoptosis, a nonmitochondria-dependent pathway, and suggested, like other authors [36], that both, mitochondrial and nonmitochondrial pathways are involved in cisplatin-induced apoptosis.

Our data showed the beneficial effect of DMTU in the prevention of cisplatin-induced apoptosis, as evidenced by the lower caspase-3 activity. The prevention of mitochondrial dysfunction (decreased ATP content, calcium uptake and electrochemical potential) by DMTU may have accounted for the protection against apoptosis, because mitochondrial functional alterations have been proposed as initial events in the apoptotic process [37, 38]. DMTU also prevented the oxidation of GSH and NADPH, therefore preventing the establishment of a pro-oxidative status and thus, protecting against lipid peroxidation (MDA + HNE), oxidation of cardiolipin and proteins. Cardiolipin oxidation is implicated in cytochrome c release to citosol, a critical early event in the mitochondrial pathway of the apoptotic cell death [8, 39]. Therefore, the prevention of cardiolipin oxidation by DMTU may also have contributed to the protection against cisplatin-induced apoptosis. Aconitase activity was also preserved by DMTU treatment. This enzyme plays a key role in the citric acid cycle (TCA), which provides electron carriers (NADH and $FADH_2$) to the oxidative phosphorylation in the respiratory chain. Furthermore, the oxidation of the aconitase iron–sulfur centers would result in Fe^{2+} release, favoring the Fenton reaction [8]. Based on that, we can hypothesize that, by protecting aconitase against oxidation, DMTU prevented impairment in ATP synthesis as well as in the Fenton reaction and in the resultant production of hydroxyl radicals, thus preventing the amplification of oxidative damage to mitochondrial proteins, DNA and lipids.

In the past few years, a new therapeutic direction, based on targeting antioxidants to mitochondria, has emerged. Strategies that exploit mitochondria-mediated cytoprotection in order to counteract the alterations in the key cellular regulatory processes including ATP production, Ca^{2+} uptake, redox state, free radical generation and apoptosis, constitute an interesting approach to design new therapies of cellular protection against chemotherapy-induced toxicity. On the basis of these aspects and considering the fact that mitochondrial injury is regarded as a central event in the early stages of the nephrotoxic effect of cisplatin, the selective protection of mitochondria against reactive oxygen species generated by cisplatin in intact tissues, such as kidney, is of critical importance in the chemotherapy of patients with cancer. By showing the important role played by the hydroxyl radicals generated in renal cells mitochondria and the effectiveness of DMTU in preventing the mitochondrial damage induced by cisplatin, our results strongly support the idea that hydroxyl radical scavenger may be an attractive target for the prevention of cisplatin-induced nephrotoxicity.

In general, natural antioxidant therapy has achieved only limited success. There are reports of slightly effective and ineffective antioxidants against cisplatin nephrotoxicity, such as curcumin [40] and glutamine [41].

Some agents have been shown to ameliorate cisplatin toxicity, but there is still a need for better antioxidants to prevent the kidney tissue damage caused by cisplatin-chemotherapy.

Ideally, antioxidants should not have any pro-oxidative effects. It has been reported that thiols produce superoxide radicals; ascorbic acid induces free radical production when coupled with a transition metal [42] and the iron chelator deferrioxamine aggravates the nephrotoxicity of cisplatin [43]. Furthermore, some in vitro studies show that vitamin A [44], vitamin C [45], genistein [46] and quercetin [47] increased the cytotoxic effects of cisplatin. The organic thiol amifostine has been used as a tissue cytoprotector in patients receiving cisplatin. Administration of amifostine has been reported to reduce cisplatin-related toxicities in some studies, but not all. Its side effects, especially transient hypotension, which probably caused the death of a patient, influence its clinical usefulness [48]. Severe neurotoxicity, ototoxicity and nephrotoxicity following high-dose cisplatin and amifostine have been reported in a girl with epithelial cell carcinoma of the ovary despite the use of amifostine [49].

In our studies, we showed that the treatment with only DMTU did not cause mitochondrial oxidative stress, alterations in the redox state or mitochondrial disfunction. In fact, the results of all the assays performed in the DMTU group remained unchanged as compared to those in the control group.

Regarding the effectiveness of cisplatin chemotherapy, DMTU is not expected to decrease it, because distinct mechanisms have been proposed. The anticancer activity of cisplatin is attributed to the formation of cisplatin-DNA adducts, which occurs after the uptake of the drug into the nucleus of cells, and which can cause various cellular responses such as: replication arrest, transcription inhibition, cell-cycle arrest, DNA repair and apoptosis [50]. On the other hand, several lines of evidence suggest that oxygen reactive species (ROS), mainly generated in mitochondria, play a central role in the cisplatin-induced renal injury [4–6]. Therefore, it appears that ROS do not play a central

role, if any, in the mechanism responsible for the antineoplastic activity of cisplatin and, in such case, the hydroxyl radical scavenger DMTU would not intervene in the anticancer action of the drug. Furthermore, it has been demonstrated that DMTU does not interact with cisplatin to form an inactive compound, which would limit the access of cisplatin to the cell and consequently decrease its anticancer activity [13]. However, additional studies are necessary to determine whether or not the antineoplastic efficacy of cisplatin would be affected by DMTU.

Some authors do not consider DMTU suitable for use in humans, because there are reports of fetotoxicity and mild lung damage in rats, at high doses [14, 51, 52]. Unlike many thioureas, symmetrical N,N′-substituted thioureas, including DMTU, are not carcinogenic and do not induce pulmonary edema. In fact, DMTU is regarded as the least toxic agent among the alkylthioureas. [53]. Dimethylthiourea (DMTU) is a small, highly diffusible molecule that effectively scavenges hydroxyl radicals, reducing oxidative injury in many biological systems. Besides that, DMTU is a potent sulfhydryl donor and has been reported to enhance renal glutathione metabolism, which might contribute to its cytoprotective property [54]. The metabolism of DMTU in vivo has not been characterized. Studies have shown in vitro that P450 enzymes and flavin monooxygenase oxidize thioureas to their corresponding S-oxides, which seem to be more toxic than the parent compounds [51, 52, 55]. In our studies, performed in vivo, DMTU did not show any direct effect on mitochondria; however, further investigations on DMTU metabolism and on the effects of the derived metabolites should be conducted in order to safely propose its clinical application. Meanwhile, our study provides evidence of the effectiveness of this agent in protecting against cisplatin-induced nephrotoxicity and delineates several mitochondrial steps by which this protection occurs. Based on that, we propose that mitochondria and the hydroxyl radical generation are, respectively, a cellular site and a target to be considered in future cytoprotective strategy. Additionally, considering the high efficiency of DMTU, we also suggest that DMTU structure might be useful in the design of novel antioxidant agents for clinical application. The combination with an adjuvant antioxidant therapy may enhance the effectiveness of cisplatin chemotherapy by ameliorating side effects and consequently enabling dose escalation.

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