BACKGROUND: Ozone therapy has become a useful treatment for pathological processes, in which the damage mediated by reactive oxygen species is involved. Several lines of evidence suggest that cisplatin-induced acute nephrotoxicity is partially mediated by reactive oxygen species.

Aims: To analyze the effect of ozone administration after cisplatin-induced acute nephrotoxicity.

Methods: Male Sprague–Dawley rats were treated with five intra-rectal applications of ozone/oxygen mixture at 0.36, 1.1 and 1.8 mg/kg after cisplatin intraperitoneal injection (6 mg/kg). Serum and kidneys were taken off 5 days after cisplatin treatment. Creatinine was measured in the serum and the activities of antioxidant enzymes and thiobarbituric acid reactive substances and glutathione content were analyzed in renal homogenate.

Results: Ozone treatment diminished the increase in serum creatinine levels, the glutathione depletion and also reversed the inhibition of superoxide dismutase, catalase and glutathione peroxidase activities induced by cisplatin in the rat kidney. Also, the renal content of thiobarbituric reactive substances was decreased by ozone/oxygen mixture applied after cisplatin.

Conclusion: Intrarectal applications of ozone reversed the renal pro-oxidant unbalance induced by cisplatin treatment by the way of stimulation to some constituents of antioxidant system in the kidney, and thereby it decreased the renal damage.

Reversion by ozone treatment of acute nephrotoxicity induced by cisplatin in rats

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Introduction

Cisplatin (CDDP) is one of the most effective chemotherapeutic agents for the treatment of ovarian, testicular and bladder carcinomas, and cancers of the head and neck and lung.[1] Unfortunately, more than 25% of patients receiving an initial dose (50–100 mg/m^2) of CDDP develop acute renal failure[1] due to its preferential accumulation within the proximal tubule cells in the outer medulla of the kidney.[2] The cellular events in CDDP-induced acute nephrotoxicity, including decreased protein synthesis, membrane lipid peroxidation, mitochondrial dysfunction and DNA injury, are a consequence of free radical generation and the inability to scavenge such molecules.[3] Furthermore, various studies have demonstrated a protective role for antioxidants and free radical scavengers such as vitamin E, lipoic acid, ebselen, superoxide dismutase (SOD), taurine, glutathione (GSH) and its esters in CDDP-induced acute nephrotoxicity.[4–9]

Very recently, studies from our laboratory have demonstrated that ozone (O$_3$) pretreatment under an oxidative preconditioning regimen for 15 days exerts protection against CDDP-induced acute renal damage in rats, and it was due to the O$_3$ protective effects on some important constituents of the antioxidant system in the kidney such as SOD, catalase (CAT), glutathione peroxidase (GSH-Px), GSH, and the concomitant reduction of renal lipid peroxidation.[10] Taking into account these findings we decided to elucidate whether ozone therapy administered after CDDP-induced acute nephrotoxicity is able to reverse it.

Materials and methods

Chemicals

Serum creatinine was measured spectrophotometrically with the creatinine assay kits purchased from Biological Products Enterprise 'Carlos J Finlay' (Havana, Cuba). All reagents used in determinations of GSH, SOD, CAT, GSH-Px, thiobarbituric acid reactive substances (TBARS) and cisplatin were purchased from Sigma Chemicals (St Louis, MO, USA). Other reagents of analytical grade were obtained from normal commercial sources.

Animals

Male Sprague–Dawley rats (200–250 g) were obtained from the National Center for Laboratory Animal Production (CENPALAB, Havana Cuba). The animals were housed under a 12 h light–dark cycle with room temperature maintained at 25°C, humidity at 60% and food and water available *ad libitum*. The experiments were conducted in accordance with the ethical guidelines for investigations in laboratory animals and were approved by the Ethical Committee for Animal Experimentation of the National Center for Scientific Research, Havana, Cuba.

Experimental design

An ozone/oxygen mixture (OOM) was generated by OZOMED 01 equipment manufactured by the Ozone Research Center (Cuba). The OOM was obtained from medical-grade oxygen and it was used immediately. The O_3 concentration was measured by an ultraviolet spectrophotometer at 254 nm.

The rats were divided into six groups of eight rats each: (1) non-treated control rats, (2) rats treated only with CDDP, (3) rats treated with CDDP plus oxygen (O_2), (4) rats treated with CDDP plus OOM (10 μg of O_3/ml at a dose of 0.36 mg/kg), (5) rats treated with CDDP plus OOM (30 μg of O_3/ml at a dose of 1.1 mg/kg), and (6) rats treated with CDDP plus OOM (50 μg of O_3/ml at a dose of 1.8 mg/kg).

CDDP (6 mg/kg) was administered to rats by an intraperitoneal injection and thereafter OOM was administered once daily by rectal insufflation during 5 days. The volume of insufflated mixture was approximately 9 ml.

Twenty-four hours after the last OOM application the rats were killed by ether overdose, and afterwards the blood was collected and serum was separated by centrifugation for creatinine analysis. The kidneys
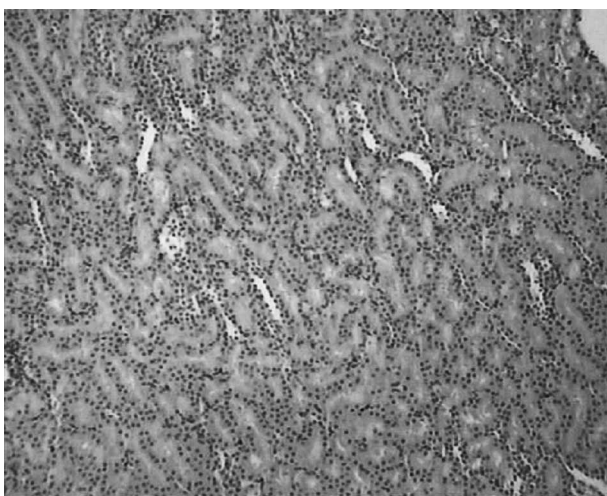
were removed and immediately frozen at $-20°C$ until biochemical and histopathological studies were performed.

Kidney homogenates were obtained using a tissue homogenator at 4°C. The homogenates were prepared with a 100 mM KCl buffer (pH 7) containing ethylenediamine tetraacetic acid 0.3 mM (1:10 w/v) for GSH, TBARS, GSH-Px and SOD determinations (Buffer 1). The homogenates were spun down with a centrifuge at $600 \times g$ for 60 min at 4°C. The supernatants were taken for biochemical determinations.

Kidney homogenates for CAT enzymatic assay were obtained with a 50 mM phosphate buffer (pH 7) containing 1% Triton X-100 (1:9 w/v) (Buffer 2). The homogenates were centrifuged at $600 \times g$ for 60 min at 4°C and the supernatants were used for the CAT assay.

Determination of GSH

GSH was determined by a slightly modified version of the method of Beutler *et al.*,[11] using a spectrophotometer. One milliliter of the kidney homogenate, as already described, was mixed with 1.5 ml of 5% metaphosphoric acid and centrifuged at $3000 \times g$ for 10 min at room temperature. Five hundred microliters of this acidic supernatant was mixed with 2 ml of 0.2 M phosphate buffer and 0.25 ml of 0.04% 5,5′-dithio-bis-2-nitrobenzoic acid. Absorbance of the yellow solution was measured at 412 nm within 10 min. A molar extinction coefficient of 13.6 M/cm that describes the formation of the thiolate anion by the reaction of sulfhydryl groups with 5,5′-dithio-bis-2-nitrobenzoic acid at 412 nm was used to quantify GSH.

Determination of SOD

The enzymatic activity of SOD was determined by a modified version of the method of Minami and Yoshikawa.[12] Fifty microliters of the kidney homogenate was mixed with 450 μl of cold deionized water, 125 μl of chloroform and 250 μl of ethanol. The mixture was centrifuged at $8000 \times g$ for 2 min at 4°C. Five hundred microliters of the extract was added to a reaction mixture containing 500 μl of 72.4 mM Tris–Cacodylate buffer with 3.5 mM diethylene pentaacetic acid (pH 8.2), 100 μl of 16% Triton-X 100 and 250 μl of 0.9 mM nitro-blue tetrazolium. The reaction mixture was incubated for 5 min at 37°C before adding 10 μl of 9 mM pyrogallol (dissolved in 10 mM hydrochloric acid), and then incubated for 5 min at 37°C. The reaction was stopped with the addition of 300 μl of 2 M formic buffer (pH 3.5) containing 16% Triton-X 100. The absorbance was measured at 540 nm on the spectrophotometer. One unit of SOD enzymatic activity is

FIG. 1. Light micrograph of the corticomedulary region of a rat kidney from the control group. Normal histology (hematoxylin and eosin, ×100).

equal to the amount of enzyme that diminishes the initial absorbance of nitro-blue tetrazolium by 50%.

Determination of CAT

CAT was determined according to the method of Rice Evans and Diplock.[13] Kidney homogenate was diluted with Buffer 2, as already described, to obtain an adequate dilution of the enzyme. Then, 2 ml of buffer 2 were added to the cuvette and mixed with 1 ml of 30 mM H_2O_2, and then the absorbance was measured at 240 nm, for 30 sec in the spectrophotometer. The initial absorbance of the reaction mixture must be around 0.5. The enzyme activity is expressed as the first-order constant that describes the decomposition of H_2O_2 at room temperature.

Determination of GSH-Px

The enzymatic activity of GSH-Px was determined using a modified version of the method of Thonson *et al.*[14] All reaction mixtures were dissolved in 20 mM sodium phosphate buffer containing 6 mM ethylene-diamine tetraacetic acid (pH 7.0). The reaction mixture consisted of 98.8 μl of phosphate buffer, 700 μl of 2.86 mM GSH, 100 μl of 1 mM sodium azide, 100 μl of 1 mM NADPH and 4.2 μl of GSH reductase (0.5 units). Then, 10 μl of the tissue homogenate supernantant were added to the reaction mixture and incubated at room temperature for 10–15 min. Afterwards, 10 μl of 30 mM *t*-butyl hydroperoxide (dissolved in bi-distilled water) was added to the reaction mixture and measured at 340 nm for 7 min in the spectrophotometer. A molar extinction coefficient of 6.22×10^3 M/cm was used to determine the activity of GSH-Px. The enzyme activity is expressed as international units of enzymatic activity/milligram of protein. International units are expressed as micro-moles of transformed hydroperoxides per minute per milliliter of enzyme.

Lipid peroxidation assay

This assay is used to determine TBARS levels as described by Ohkawa *et al.*[15] Two hundred milliliters of tissue homogenate supernatant were added to 100 μl of sodium dodecyl sulfate, 750 μl of 20% acetic acid (pH 3.5), 750 μl of 0.6% thiobarbituric acid and 300 μl of distilled water and were incubated at 95°C for 60 min. The samples were allowed to cool at room temperature. Then 2.5 ml of butanol:pyridine (15:1) and 500 μl of distilled water were added, vortexed, and centrifuged at $2000 \times g$ for 15 min. The absorbance of 3 ml of the colored layer was measured at 532 nm spectrophotometrically using 1,1,3,3-tetraethoxypropane as the standard.

Protein assay

Protein concentrations were determined by the method of Lowry *et al.*[16] using bovine serum albumin as standard.

Histopathological assessment of renal damage

The left kidneys were quickly removed and fixed in 10% formaldehyde. Tissues were embedded in paraffin, sectioned at 3 μm, stained with hematoxylin and eosin and evaluated by light microscopy.

Statistical analysis

Data are expressed as the mean $\pm$ standard error of the mean and analyzed statistically using one-way analysis of variance followed by the Duncan multiple range test for serum creatinine determinations, whereas the Kruskall–Wallis test followed by the Mann–Whitney test was applied for the rest of the markers. The 0.05 level of probability was used as statistical significance.

Results

Serum creatinine levels significantly increased in CDDP-injected rats as compared with non-treated control ($p < 0.01$). Graded doses of OOM (0.36, 1.1 and 1.8 mg/kg) significantly reduced in 50% the increase of serum creatinine levels as compared with CDDP alone and CDDP plus O_2, indicating that O_3 treatment ameliorated the nephrotoxicity of CDDP (Table 1).

Also, O_3 treatment induced reversion of the renal GSH depletion induced by CDDP. This effect was significantly greater at an O_3 dose of 1.1 mg/kg as compared with CDDP-treated or non-treated rats. In agreement with that finding, the OOM significantly reduced, the renal TBARS content at an O_3 dose of 1.1 mg/kg, although the other two tested doses did not induce any significant change with respect to CDDP-treated control rats.

The SOD activity in the kidney significantly decreased (44% of control) in CDDP-treated rats. The treatment with OOM induced reversion of SOD activity up to values very close to those of the non-treated control group. Thus, O_3 treatment exerted a stimulating effect in renal SOD activity as compared with CDDP alone. This effect was dose dependent.

CAT activity in the kidney significantly decreased (48% of control) in CDDP-treated rats as compared with the control group. The treatment with OOM induced also in a dose-dependent fashion a significant reversion of that effect. The increase of renal CAT activity by O_3 treatment was greater with doses

R. González et al.

Table 1. Effect of ozone treatment on biochemical markers evaluated in CDDP-induced nephrotoxicity in rats

Experimental group	Serum creatinine levels (μM)	GSH (nmol/mg of protein)	TBARS (nmol/mg of protein)	SOD (SOD units/mg of protein)	CAT (k_{15}/g of wet tissue)	GSH-Px (UI/mg of protein)
Non-treated control	62.3±11.98[a]	9.3±0.90[a]	0.2±0.03[a]	8.6±1.21[a]	6.8±0.39[a]	7.4±1.28[a]
CDDP control	207.2±98.11[b]	4.8±0.82[b]	0.6±0.12[b]	4.8±1.22[b]	3.5±0.50[b]	5.6±1.47[b]
CDDP control+O_2	216.8±18.92[b]	5.5±1.64[b]	0.7±0.17[b]	4.4±1.01[b]	3.8±0.51[b]	5.7±0.79[b]
CDDP + 0.36 mg/kg O_3	91.2±23.30[c]	6.7±1.99[c]	0.5±0.05[b]	6.6±1.07[c]	4.8±1.08[c]	6.3±1.64[b]
CDDP + 1.1 mg/kg O_3	104.7±8.54[c]	12.5±3.47[d]	0.3±0.09[c]	8.8±1.41[a]	6.6±0.55[a]	7.9±0.8[a]
CDDP + 1.8 mg/kg O_3	101.6±19.01[c]	7.9±1.16[c]	0.5±0.07[b]	8.9±1.33[a]	6.5±1.3[a]	7.4±1.91[a]

Different letters means significant differences between groups ($p < 0.05$). Each value represents means±standard error of the mean for seven animals per group. One unit of SOD enzymatic activity is equal to the amount of enzyme that diminishes the initial absorbance of nitro-blue tetrazolium by 50%, and CAT activity is described as the first-order constant of the decomposition of H_2O_2 at 25°C/g of wet tissue. The GSH-Px activity is expressed as international units (UI) of enzymatic activity/milligram of protein. International units are expressed as micromoles of transformed hydroperoxides per minute per milliliter of enzyme.

of 1.1 and 1.8 mg/kg as compared with CDDP or CDDP plus O_2 treatment, and was very close to values of the non-treated control group.

Also, GSH-Px activity in the kidney was significantly decreased in CDDP-treated rats with respect to non-treated rats. O_3 treatment also significantly increased GSH-Px activity as compared with CDDP alone. O_3 doses of 1.1 and 1.8 mg/kg also induced values of enzyme activity that reached those of non-treated rats.

The histopathological changes observed in the kidneys in this experiment revealed that in rats treated with CDDP alone, severe and widespread tubular necrosis with dilation of proximal tubules, dequamation of renal tubular cells, and cast formation in the lumen was observed. Characteristically, the tubular lesions were mostly localized in the corticomedulary region 5 days after CDDP administration (Fig. 2).

In contrast, in rats treated with CDDP and thereafter with five i.r. applications of OOM (1.1 mg/kg of O_3) the tubular necrosis was slight, and in a lesser extent than it occurred in rats treated with CDDP alone. Tubular dilation and cast formation in the tubular lumen were also reduced in rats treated with O_3 (Fig. 3)

Discussion

To date different strategies have been proposed to inhibit CDDP-induced nephrotoxicity. One of them is the use of antioxidant therapies to prevent the generation of reactive oxygen species, which exert
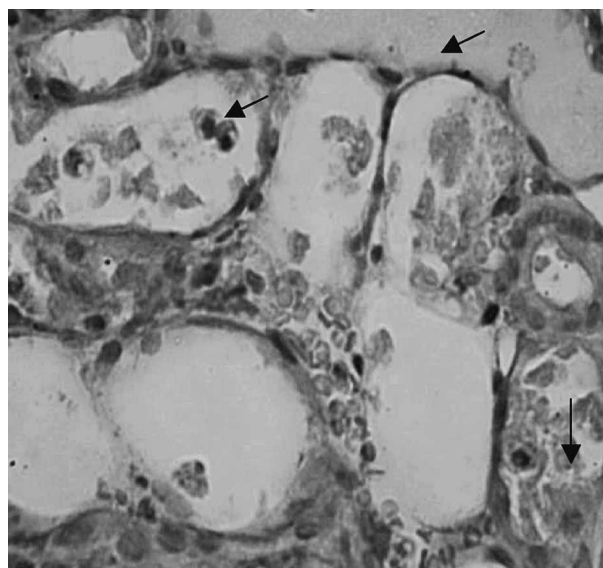

FIG. 2. Light micrograph of the corticomedulary region of a rat kidney treated with CDDP alone. Typical severe tubular necrosis with dilation of proximal tubules is shown. Arrows indicate dequamation of renal tubular cells and cast formation in the tubular lumen (hematoxylin and eosin, ×400).
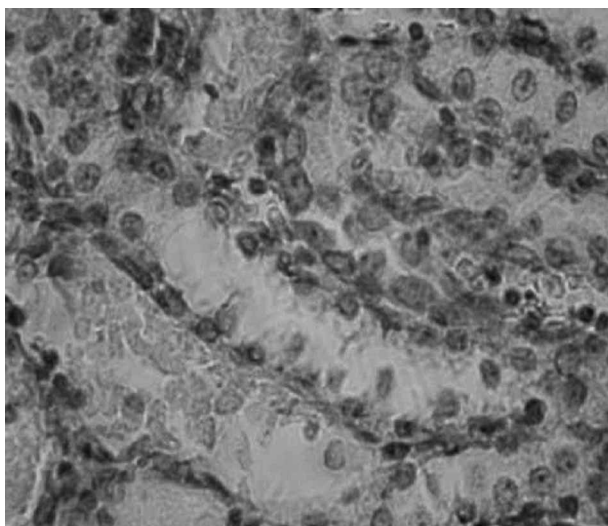
FIG. 3. Light micrograph of the corticomedulary region of a rat kidney treated with OOM after CDDP injection. Tubular necrosis was slight and in lesser extent than in Fig. 2. Note the significant diminution in the dequamation of tubular epithelial cells (hematoxylin and eosin, ×400).

an influence on the progression of oxidative renal damage induced by CDDP.[17] Thus, a proposal has been the use of enriched diets with natural antioxidants like vitamin E, ascorbic acid and methionine among others.[4,18] Another proposal has been the use of sulfhydryl-containing drugs such as diethyldithiocarbamate, sodium thiosulfate, N-acetylcysteine and lipoic acid, which also exert antioxidant activity.[5]

However, although in animal models all these compounds have shown protective effects in CDDP-induced acute nephrotoxicity, none of them has proved to be clinically efficacious as a complete protective agent in patients.[5]

Very recently, it was demonstrated in our laboratory that O_3 pretreatment under an oxidative preconditioning regimen for 15 days exerted protective effects on some important constituents of the endogenous antioxidant system in the kidney such as SOD, CAT, GSH-Px and GSH in CDDP-induced acute nephrotoxicity.[10] Our present results also demonstrate that i.r. O_3 therapy for 5 days after CDDP administration attenuates its nephrotoxicity and also restores the levels of antioxidant defense constituents (GSH, SOD, CAT and GSH-Px) depressed by CDDP up to values close to those of non-treated control rats (Table 1).

Furthermore, the stimulant effects of O_3 therapy on antioxidant system were accompanied by a decrease in both serum creatinine levels and renal TBARS content, the latter used as marker of lipid peroxidation in the kidney. Thus, all these aforementioned effects of O_3 explain by which mechanisms attenuated acute renal damage is induced by CDDP.

Induction of CAT, SOD and GSH-Px by ozone therapy is probably due to H_2O_2 produced as result of O_3 decomposition, because it is one of the major O_3 intermediates along with $OH^\bullet$ and O^{z-}. Whiteside and Hassan[19] also demonstrated induction of CAT and SOD by O_3 in cultures of *Escherichia coli*. Furthermore, they showed that an increase in the activities of CAT and SOD by O_3 was due to induction of the novo enzyme synthesis rather than activation of pre-existing apoproteins.

Induction of CAT and SOD might be related with activation of gene/regulatory nuclear factor-kappa B (NF-κB) by H_2O_2. This transcription factor appears to play many roles in stress responses, inflammation, cell cycle regulation and apoptosis,[20] and it has been closely related with the release of cytokines after ozonation of blood *ex vivo*.[21] However, we found no nuclear expression of NF-κB 5 days after cisplatin injection in rats (unpublished results), which might suggest that NF-κB is not directly related with the induction of antioxidant enzymes observed in this experiment, but may be influencing the diminution in inflammatory responses induced by this drug in renal tissue[22] detected after the fifth day in ozone-treated rats previously injected with cisplatin (unpublished data).

Other proteins that might be involved in ozone-induced recovery in cisplatin nephropathy are the Bcl-2 family of proto-oncogenes (unpublished results). Within 5 days after cisplatin administration, levels of pro-apoptotic Bax mRNA were significantly increased, as reported by other authors,[23] and we detected a significant decrease in the expression of this protein with five applications of ozone after cisplatin injection, which is correlated with an increase in the expression of Bcl/X_L (unpublished data), favoring the survival and regeneration of the renal tissue.

Thus, the induction of SOD, CAT and GSH-Px in response to O_3 treatment in CDDP-induced nephrotoxicity provides further evidence that there is a correlation between antioxidant enzyme biosynthesis and O_3 exposure, which supports the potential usefulness of this therapy in the prevention and treatment of this toxic nephropathy.

Rectal insufflation of O_3 is a simple procedure and it was reported free of side effects in humans; however, the accurate measurement of the O_3 dosage is difficult to assess,[24] although it is the most useful and easy method to perform in rats. Autohemotherapy, in which a volume of blood is extracted and exposed to a precise ozone dose, represents the most suitable method of application in humans, because there is a reasonable stoichiometric relationship between O_3 and blood.[25] Therefore, autohemotherapy might be promissory for the treatment of cisplatin nephropathy.

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