**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. Unfortunately, more than 25% of patients receiving an initial dose (50–100 mg/m²) of CDDP develop acute renal failure due to its preferential accumulation within the proximal tubule cells in the outer medulla of the kidney. 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.

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. Very recently, studies from our laboratory have demonstrated that ozone (O₃) 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 O₃ 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. 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 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 ethylenediaminetetraacetic 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 × 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 × 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., 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 × 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$^{-1}$ 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. 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 × 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 diethyl 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
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.\textsuperscript{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$_2$O$_2$, 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$_2$O$_2$ at room temperature.

Determination of GSH-Px

The enzymatic activity of GSH-Px was determined using a modified version of the method of Thonson \textit{et al.}\textsuperscript{14} All reaction mixtures were dissolved in 20 mM sodium phosphate buffer containing 6 mM ethylenediamine tetraacetic acid (pH 7.0). The reaction mixture consisted of 98.8 $\mu$l of phosphate buffer, 700 $\mu$l of 2.86 mM GSH, 100 $\mu$l of 1 mM sodium azide, 100 $\mu$l of 1 mM NADPH and 4.2 $\mu$l of GSH reductase (0.5 units). Then, 10 $\mu$l of the tissue homogenate supernatant were added to the reaction mixture and incubated at room temperature for 10–15 min. Afterwards, 10 $\mu$l 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 $\times$ 10$^3$ M$^{-1}$cm$^{-1}$ 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 micromoles of transformed hydroperoxides per minute per milliliter of enzyme.

Lipid peroxidation assay

This assay is used to determine TBARS levels as described by Ohkawa \textit{et al.}\textsuperscript{15} Two hundred milliliters of tissue homogenate supernatant were added to 100 $\mu$l of sodium dodecyl sulfate, 750 $\mu$l of 20% acetic acid (pH 3.5), 750 $\mu$l of 0.6% thiobarbituric acid and 300 $\mu$l 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 $\mu$l 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 \textit{et al.}\textsuperscript{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 $\mu$m, stained with hematoxylin and eosin and evaluated by light microscopy.

Statistical analysis

Data are expressed as the mean±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

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of 1.1 and 1.8 mg/kg as compared with CDDP or CDDP plus O₂ 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. O₃ treatment also significantly increased GSH-Px activity as compared with CDDP alone. O₃ 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, degeneration of renal tubular cells, and cast formation in the lumen was observed. Characteristically, the tubular lesions were mostly localized in the corticomedullary region 5 days after CDDP administration (Fig. 2).

In contrast, in rats treated with CDDP and thereafter with five i.r. applications of O₃ (1.1 mg/kg of O₃) 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 O₃ (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).](image-url)
Induction of CAT, SOD and GSH-Px by ozone therapy is probably due to H$_2$O$_2$ produced as result of O$_3$ decomposition, because it is one of the major O$_3$ intermediates along with OH$^*$ and O$_2^*$. 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-kB) by H$_2$O$_2$. 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*. However, we found no nuclear expression of NF-kB 5 days after cisplatin injection in rats (unpublished results), which might suggest that NF-kB 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$\text{XL}$ (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 promising for the treatment of cisplatin nephrotoxicity.
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