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Effects of Ozone Therapy on Cyclophosphamide-induced Urinary Bladder Toxicity in Rats

Abstract

Purpose: This study investigated the efficacy of ozone therapy (OT) in a rat model of cyclophosphamide-induced hemorrhagic cystitis (HC).

Methods: Forty Wistar Albino male rats were divided into five groups: sham, OT, cyclophosphamide (CP), OT+CP and CP+OT. Hemorrhagic cystitis (HC) was induced by intraperitoneal (i.p) administration a single dose of 100 mg/kg CP. OT was performed once daily for three days. The CP+OT group received OT (0.2 mg/kg) i.p 24 h after CP administration. CP was injected to the OT+CP group the day after the third course of OT. All animals were killed four days after CP administration. Bladder injury and oxidative stress parameters were determined from tissue samples.

Results: We found small, but non-statistically significant biochemical and histological changes in the animals treated with OT alone. CP administration induced cystitis, as manifested by a marked loss of urothelial cells, as well as hemorrhaging and edema in the bladder as determined by histopathological examination. It also caused a significant decrease in the endogenous antioxidant compound glutathione (GSH) and elevation of lipid peroxidation, and nitric oxide (NO) and myeloperoxidase (MPO) levels in the rats' urinary bladder tissue. OT was able to ameliorate these changes; however these effects were prominent in the CP+OT group when compared with the OT+CP group.: For example, the NO level in the CP+OT group was 68% of the OT+CP group ($p < 0.05$).

Conclusion: OT prevented CP-induced urothelial damage by diminishing bladder oxidative stress, inflammation and NO levels. OT may help to ameliorate bladder damage induced by CP in the clinical setting.

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Cyclophosphamide (CP), a cytotoxic alkylating agent, is used at high doses for the treatment of a number of cancers including lymphoma, leukemia, bronchial, breast and ovarian cancers, at low doses for the treatment of autoimmune diseases and also as an immunosuppressant after organ transplantations [1].

Hemorrhagic cystitis (HC) is a known complication of high-dose cyclophosphamide treatment and is a major potential toxicity and dose-limiting side effect of CP. Its clinical symptoms range from transient irritative voiding symptoms, such as urinary frequency, dysuria, urgency, suprapubic discomfort and strangury with microhematuria, to life-threatening HC. It has also been reported that patients with massive bladder hemorrhaging have bladder fibrosis, necrosis, contracture, vesicoureteral reflux and a 4% mortality rate [2]. Previous studies have suggested that nitric oxide (NO), a short-lived and highly reactive free radical, may play an important role in the pathogenesis of cyclophosphamide-induced HC [3–5]. NO is produced from the amino acid L-arginine by the enzyme NO synthase (NOS), and it regulates a number of important physiological and pathophysiological processes including vascular tone, polymorphonuclear leukocytes, adhesion and inflammation [6].

Acrolein, a metabolic product of CP, rapidly reacts at many cellular sites and depletes the cellular thiol, glutathione. It can also react with some protein residues and with nucleophilic sites in DNA. Additionally, it has also been identified as an initiator of lipid peroxidation [7]. Acrolein enters rapidly into the uroepithelium because of its chemical nature (an unsaturated aldehyde) and causes increased reactive oxygen species (ROS) production in the bladder epithelium. Numerous studies using several antioxidants, such as α -tocopherol [8], β -carotene [9] and melatonin [10,11], have investigated whether the scavenging of ROS with antioxidants may ameliorate HC symptoms. Such studies have found that the antioxidants had protective effects against CP-induced bladder damage.

Ozone has been used as a therapeutic agent for the treatment of different diseases [12,13]. Recently, studies have shown that OT exhibits useful effects in conditions ranging from peritonitis, infected wounds, chronic skin ulcers, initial gangrene, burns and advanced ischemic diseases [14]. Di Filippo et al. reported that pre-treatment with ozone had a protective effect on acute myocardial infarction, likely through the modification of the oxidative, inflammatory, immune and apoptotic responses within the myocardium [15]. It was also demonstrated that ozone increased antioxidant enzyme activities, such as glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) [16–18]. Additionally, Koca et al. suggested that the OT reduced oxidative/nitrosative status and inducible

NO synthase (iNOS), produced NO in skeletal muscles [19]. Therefore, the aim of the present study was to investigate the potential protective effect of OT on CP-induced cystitis in rats.

Materials and Methods

Experimental conditions

In this study, 40 male Wistar Albino rats (weighing 250–350 g) were obtained from the Experimental Animal Research Center, Medical Faculty, Inonu University, Malatya, Turkey. The animals were kept in a temperature- (21 ± 2 °C) and humidity-controlled room ($60 \pm 5\%$) in which a 12 h/12 h light–dark cycle was maintained. Animals had free access to food and water. The experiment was performed in accordance with the Guidelines for Animal Research from the National Institute of Health and was approved by the Committee on Animal Research at Inonu University, Malatya, Turkey.

Experimental groups

Ozone was generated from medical-grade oxygen (O₂) using electrical corona arc discharge and the O₃ generator (model OZONOSAN Photonik 1014, Hansler, Iffezheim, Germany) that allows the gas flow rate and ozone concentration to be controlled in real time by photometric determination, as recommended by the Standardisation Committee of the International Ozone Association.

The animals were randomly divided into five groups. Each group contained eight animals as follows:

Group I (Sham): Animals were injected with 2 ml of saline (i.p.).

Group II (OT): Ozone was administered to the rats at doses of 0.2 mg/kg (i.p) [20]. OT was performed once daily for 3 days. The volume of each ozone dose was 2 ml.

Group III (CP): HC induction was performed by a single injection of CP (Endoxan, Eczacibasi-Baxter, Istanbul, Turkey) at doses of 100 mg/kg in 2 ml saline (i.p) [21].

Group IV (OT+CP): CP was injected the day after the third course of OT.

Group V (CP+OT): OT was administered for three days starting 24 h after CP injection.

Four days after CP injection, rats were anesthetized using ketamine HCl (85 mg/kg) and xylazine HCl (12.5 mg/kg) i.p. The bladders were removed intact, evacuated of residual urine and cleaned from the connective and lipid tissues surrounding the walls. Then, the bladders were cut into two equal pieces from dome to bottom and processed for histological and biochemical analyses.

Biochemical determination

Tissues were homogenized (PCV Kinematica Status Homogenizator, Littau-Luzern, Switzerland) in ice-cold phosphate buffered saline (pH 7.4). The homogenate was sonified with an ultrasonifier (Bronson sonifier 450) for 3 cycles (20 s sonications and 40 s pause on ice). The homogenate was centrifuged (15,000 \times g, 10 min, 4°C) and cell-free supernatant was subjected to an enzyme assay immediately.

The level of GSH was performed using the method of Theodorus *et al.* [22] with several modifications. The reaction mixture contained 50 mM of sodium phosphate, 1 mM of EDTA, 0.5 mM of DTNB, 0.2 mM of NADPH and 0.5 U/mL of glutathione reductase. Homogenate (10 μ L) was added to initiate the reaction but was omitted for control. The formation of 2-nitro-5-thiobenzoate was followed spectrophotometrically at 412 nm. The amount of GSH in the extract was determined as nmol/mg of protein utilizing commercially available GSH as the standard.

Tissue-associated MPO activity was measured using a procedure similar to that documented by Hillegas *et al.* [23]. Bladder samples were homogenized in 50 mmol/L of a potassium phosphate buffer (PB, pH 6.0) and centrifuged at 41400 g (10 min); pellets were suspended in 50 mmol/L of PB containing 0.5% hexadecyltrimethylammonium bromide (HETAB). After three freeze-thaw cycles, with sonication between cycles, the samples were again centrifuged at 41400 g for 10 min. Aliquots (0.3 ml) were added to 2.3 ml of the reaction mixture containing 50 mmol/L of PB, o-dianisidine, and 20 mmol/L of an H₂O₂ solution. One unit of enzyme activity was defined as the amount of MPO that caused a 3 minute change in absorbance, measured at 460 nm. MPO activity was expressed as U/mg protein.

The analysis of lipid peroxidation was carried out as described by Buege and Aust [24] with a minor modification. The reaction mixture was prepared by adding 250 μ L of homogenate into 2 ml of the reaction solution (15% trichloroacetic acid: 0.375% thiobarbituric acid: 0.25 N HCl, 1:1:1, w/v) and heated at 100°C for 15 min. The mixture was cooled to room temperature, centrifuged (10000 g for 10 min), and then the absorbance of the supernatant was recorded at 532 nm. 1,1,3,3-tetramethoxypropane was used as a Malondialdehyde (MDA) standard. MDA results were expressed as nmol mg⁻¹ of protein in the homogenate.

–Nitric oxide (NO) and nitrogen dioxide (NO₂) (NO_x) levels of the tissue samples were measured using the Cayman Nitrate/Nitrite Fluorometric Assay Kit (Ann Arbor, Michigan, USA). This kit provides a convenient method for the quantitation of NO₂/NO₃ in biological samples: the final products of

NO production *in vivo*. Therefore, the best index of total NO production is the sum of both of these products. The results were expressed as mmol/mg protein.

Protein levels of the tissue samples were measured using the Bradford method [25]. The absorbance measurement was taken at 595 nm using a UV-VIS spectrophotometer. Bovine serum albumin (BSA) was used as the protein standard.

Histological analysis

The tissues were fixed in 10% formalin and embedded in paraffin. Tissue sections were cut at 5 μ m, mounted on slides and stained with hematoxylin-eosin (H-E) to obtain the general structure of the urinary bladder. The sections were examined using a Leica DFC 280 light microscope for overall histological examination. The bladder damage severity was semi-quantitatively assessed as follows: edema, hemorrhage, desquamation of epithelial cells and destruction of smooth-muscle cells. Microscopic damage was identified as follows: 0, absent (normal appearance); 1, slight (all parameters were minimal); 2, moderate (the parameters were observed just in local areas), and 3, severe (the parameters were widespread), for each parameter measured.

Statistical analysis

Statistical analysis was performed with the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL), version 15.0. The data were initially tested for normal distribution using the Shapiro-Wilk test and were found to be abnormal ($p < 0.05$). The histological results were compared with Kruskal-Wallis variance analysis. Where differences among the groups were detected, group means were compared using the Mann-Whitney U test. The biochemical data were analyzed using a one-way analysis of variance (ANOVA) and Tukey's *post hoc* test. $p < 0.05$ was recognized as statistically significant in all the analyses.

Results

Histological evaluation

The histological structure of the stratified transitional epithelium, underlying lamina propria and muscular layers were normal in the control group (Fig. 1A). The histology of the OT group was similar to the sham group, except slight congestion and slight edema were present in the OT group (Fig. 1B). On the other hand, rats receiving CP showed severe pathologic changes, with a loss of urothelial cells and hemorrhaging. Additionally, separation of the collagen fibers was observed in the

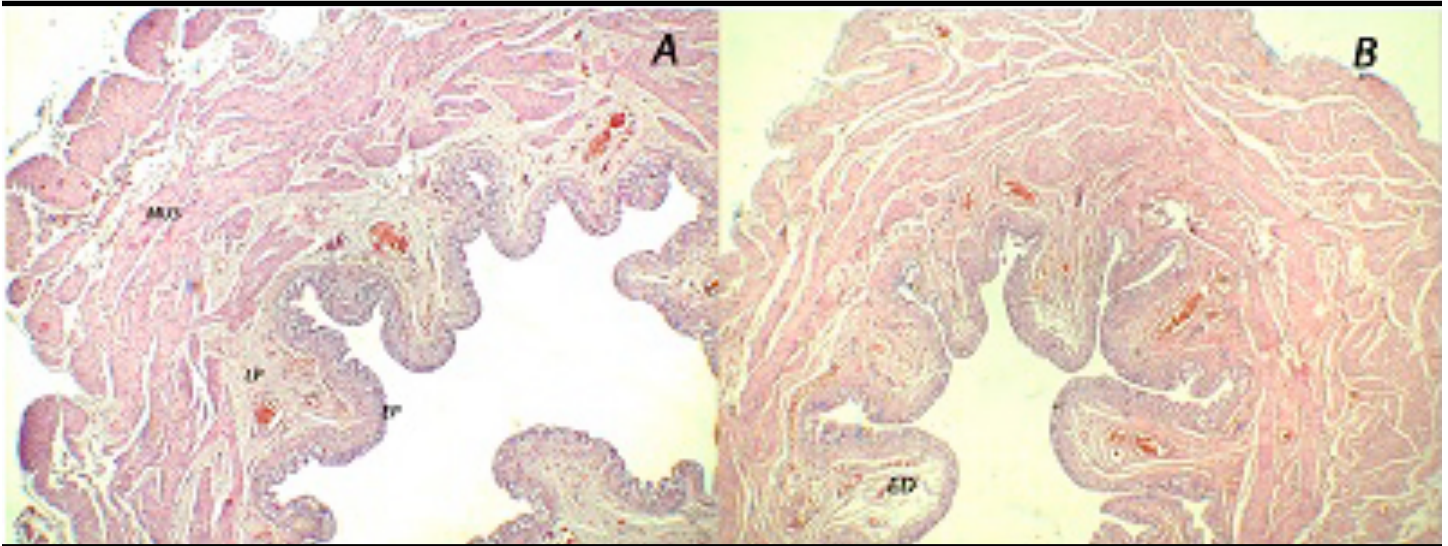


FIGURE 1. (A) Sham group Appearance of transitional epithelium (EP), underlying lamina propria (LP) and layer of muscle smooth cells (MUS). (B) OT group; slight edema is present (ED). H-E X4

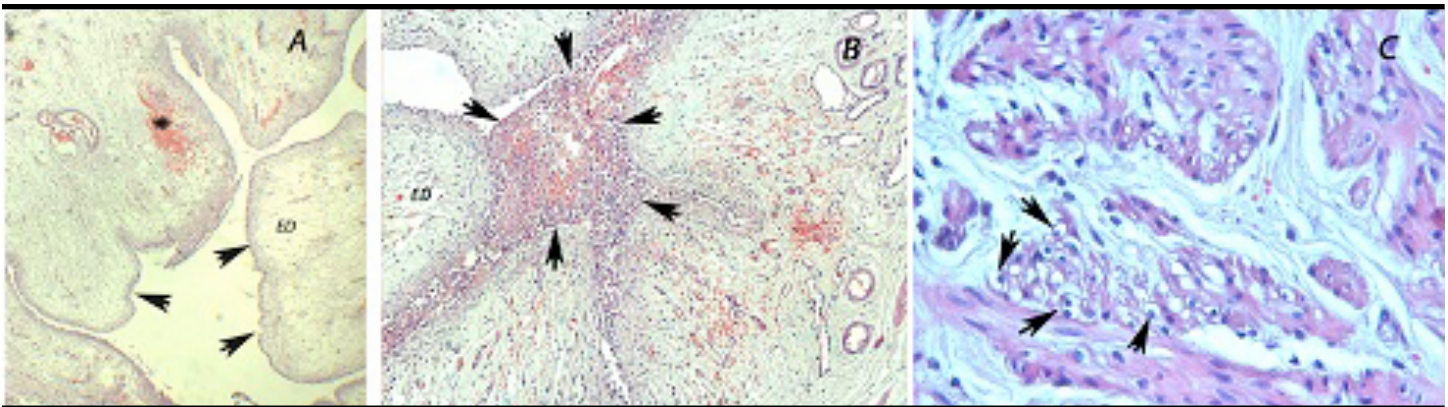


FIGURE 2. CP group (A) Hemorrhage (*) severe epithelial desquamation (arrows) and edema (ED) in the lamina propria are present. (B) polymorphonuclear leukocytes and degenerate urothelial cells (arrows) are visible in the lumen of the urinary bladder. Edema (ED) is evident. (C) Degenerative changes in smooth muscle cells are seen (arrows) H-E X4, X10 and X40.

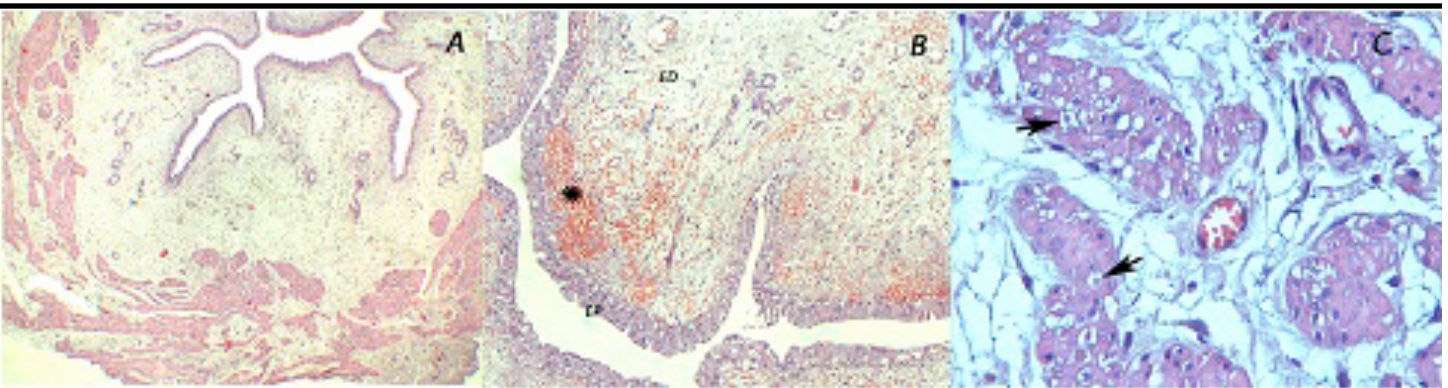


FIGURE 3. OT+CP group (A) View of histological structures of urinary bladder, (B) Transitional epithelium (EP) is intact, hemorrhage (*) and edema (ED) are visible. (C) Vacuolization in the smooth muscle cell (arrows). H-E X4, X10 and X40.

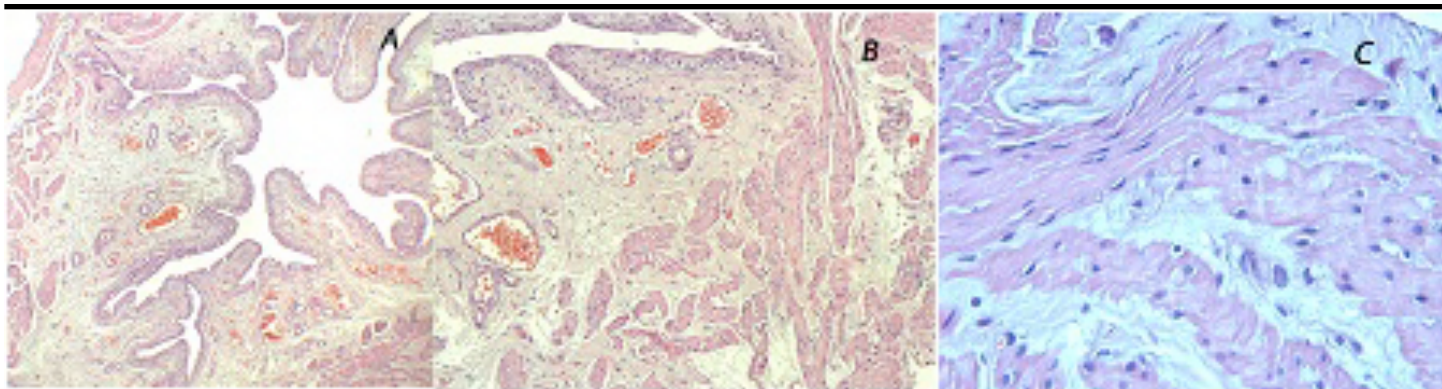


FIGURE 4. CP+OT group (A) Appearance of general histological structures. (B) Slight edema is present. (C) Smooth muscle cells are almost normal. H-E X4, X10 and X40

TABLE 1. Effect of OT on CP-induced microscopic damage

Parameters	Sham	OT	CP	OT +CP	CP+OT
Microscopic damage	0.1 ± 0.1	0.8 ± 0.4	9.0 ± 0.5 ^a	5.5 ± 0.3 ^b	2.7 ± 0.3 ^{b,c}

Microscopic damage determined semi-quantitatively using...

OT, ozone therapy; CP, cyclophosphamide

^aSignificantly increased when compared with sham group, $p=0.001$.

^bSignificantly decreased when compared with CP group, $p=0.002$

^cSignificantly decreased when compared with OT+CP group, $p=0.001$

TABLE 2. Mean MPO, NO, MDA and GSH values of the groups (Mean values ± SE)

Groups	MPO (U/mg protein)	NO (mmol/mg protein)	MDA (nmol mg ⁻¹)	GSH (nmol/mg)
Sham	0.60±0.05	124.47±11.0	0.39±0.02	58.02±3.53
OT	0.65±0.14 ^d	149.43±10.17 ^d	0.46±0.03 ^d	61.23±3.98 ^d
CP	1.07±0.12 ^a	263.46±11.88 ^a	0.79±0.07 ^a	31.27±2.84
OT+CP	0.98±0.08 ^b	204.70±13.88 ^b	0.59±0.03 ^b	48.04±2.69 ^c
CP+OT	0.90±0.13 ^b	138.65±9.94 ^{b,c}	0.48±0.03 ^b	52.95±3.95 ^c

MPO, myeloperoxidase; NO, nitric oxide; MDA, Malondialdehyde; GSH, glutathione

^aSignificantly increased when compared with sham group, $p<0.05$.

^bSignificantly decreased when compared with CP group, $p<0.05$

^cSignificantly increased when compared with CP group, $p<0.05$

^dNot significant when compared with sham group, $P > 0.05$.

^eSignificantly decreased when compared with OT+CP group, $p<0.05$

connective tissues due to a severe degree of edema (Fig. 2A). Polymorphonuclear leukocytes, together with exfoliated urothelial cells, were detected in the lumen of the urinary bladder in the CP group (Fig. 2B). Moreover, the smooth muscle of the urinary bladder wall showed damage. Affected cells dis-

played degenerative changes such as large vacuoles within the cytoplasm around the nuclei (Fig. 2C).

The microscopic damage score for each group was determined and the results are given in Table 1. The histological damage of the urinary bladder was ameliorated in the OT+CP (5.5±0.3) and CP+OT (2.7±0.3) groups compared with the

CP group (9.0 ± 0.5). Although the urothelium maintained a nearly normal appearance, hemorrhaging, edema and damaged smooth muscle cells were found in several areas, but the damage was not as extensive as that seen in the CP group (Fig. 3 vs 4). Treatment with OT following CP injection was more effective than treatment with OT prior to CP injection. The CP+OT group showed only slight degeneration of the connective tissue relative to the sham group or OT alone.

Biochemical results

The effects of CP and OT on the biochemical parameters are listed in Table 2. CP injections resulted in increased MDA, MPO, and NO levels and decreased GSH levels, indicating that oxidative stress was present in the bladders. OT alone caused only slight biochemical changes in the urinary bladders. Pretreatment with ozone (OT+CP) ameliorated the undesirable CP-induced effects, as indicated by a significant decrease in lipid peroxides (as reflected the production of MDA), MPO, NO and a marked increase in antioxidant (GSH) levels. The most prominent amelioration was detected in the CP+OT group. In particular, the level of NO was significantly lower in the CP+OT group than the OT+CP group, ($p < 0.05$) and comparable to levels seen in the sham and OT groups.

Discussion

CP is a widely used antineoplastic agent, but its side effect, HC, is still a major problem. The frequency of HC is related to dosage, and it occurs in 2–40% of patients treated with high-dose CP. In accordance with existing knowledge, several transcription factors and cytokines (tumor necrosis factor, the interleukin family and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), free radicals (ROS and NO production) and poly adenosine diphosphate-ribose polymerase activation play a role in its pathogenesis. There is also no doubt that CP-induced HC is an inflammatory process [26].

Endogenous NO regulates a number of important physiological and pathophysiological processes. Endothelial NOS (eNOS) is found in endothelial cells and fibroblasts, and it is mainly responsible for vasodilation. Neuronal NOS (nNOS) is found in the nervous system where it acts as an important signaling molecule. On the other hand, inducible NOS (iNOS) can be upregulated in nearly all cell types, including macrophages, fibroblasts and epithelial cells, and its elevated levels result in the production of large amounts of NO. It has also been suggested that NO produced by iNOS is toxic, since selective iNOS inhibition has been found to improve the outcome and decreases inflammatory events in animal models

[19,27]. There are numerous reports documenting the role of NO in CP-induced HC [6,9,28]. Additionally, it has been shown that bladder damage may be due not only to the overproduction of NO but also of ROS [29–31]. It is well known that reactions of ROS with biomolecules such as lipids can initiate chain reactions and lead to tissue damage [32]. Furthermore, CP produces a decrease in the endogenous antioxidant compound, glutathione [33,34]. Our findings are consistent with those of previous studies finding a significant reduction of GSH levels and a significant increase in lipid peroxides (MDA) and NO levels in bladder tissue after a single CP injection. GSH is one such non-enzymatic antioxidant and serves both as an electron donor for glutathione peroxidase and as a strong reducing agent to maintain intracellular redox balance [35].

Myeloperoxidase (MPO) is a hemoprotein characterized by powerful pro-oxidative and pro-inflammatory properties. It is stored in azurophilic granules of polymorphonuclear neutrophils and macrophages and released into extracellular fluid during inflammatory processes [36]. HC is now accepted as a non-microbial inflammation, and the pathogenesis of urothelial damage appears to depend on cytokine production such as IL-1 β and TNF- α leading to nitric oxide synthase (iNOS) induction [29,33]. Likewise, our findings indicated that CP caused a significant increase in the MPO level, a marker of inflammation and oxidative stress.

Ozone was first used as a treatment for infections during World War I, with successful results [19]. OT was recently shown to have protective effects against pathologies including ischemic injury, cardiopathy, atherosclerosis, and septic shock [12,16,37–40]. The efficacy of ozone in treatment is thought to result from modification of the oxidative, inflammatory, immune, and apoptotic responses [15]. Several pieces of experimental evidence have reported that OT changed levels of inflammatory cytokines, such as tumor necrosis factor- α [41], transforming growth factor- β [42], interferon [43] and interleukin (IL)-8 [44]. In addition, it has been demonstrated that OT supports cellular antioxidant systems involving glutathione, SOD, and catalase and it reduces tissue lipid peroxidation, nitrosative stress and iNOS [45,46]. Koca *et al.* reported that ozone pre-treatment has a protective effect during acute myocardial infarction. This is most likely due to its oxidative preconditioning mechanism, similar to ischemic preconditioning, which protects the organs from the damage produced by ROS, thus improving the antioxidant-pro-oxidant balance and the concomitant preservation of the cell redox state [19]. In addition, Di Filippo *et al.* indicated that the protective effect of OT was closely related to the increase in cardiac eNOS expression that results in significant increases in endothelial pro-

genitor cell levels within the myocardium and improved molecular alterations following myocardial infarction [15]. The present study showed a decrease in GSH levels and the rise in MPO, NO, and MDA levels in the animals treated with CP alone were almost entirely prevented by co-treatment of the rats with ozone; however, these effects were more prominent in the CP+OT group than the OT+CP group. Notably, NO levels in the CP+OT group were significantly lower than those in the OT+CP group. Increased NO production is most likely responsible for the cystitis because S-methylisothiourea (an iNOS-selective inhibitor) nearly abolishes CP-induced bladder damage [47,48]. Moreover, Korkmaz *et al.* indicated that CP markedly increased iNOS activity in the bladder and NOS inhibition significantly reduced the mucosal damage, hemorrhage edema, and leukocyte infiltration [26]. NO can induce both cellular cytotoxicity and tissue injury via lipid peroxidation, DNA damage and pro-apoptotic effects and a protective effect in vasodilatation, anti-apoptotic action, inhibition of platelet plug formation and reduction of the inflammatory response [49,50]. Thus, the cellular effects of NO may depend on its concentration, site of release and duration of action [49,51]. These findings are in agreement with those of the present study, in which the protective effect of OT was maximum in the CP+OT group, which had a significant depletion of NO and a significant increase in GSH. A slight increase was detected in NO, MDA and MPO levels in the OT group suggests changes consistent with mild inflammatory effects. That ozone, one of the most potent oxidizers, may induce an antioxidant response capable of reversing a chronic oxidative stress may, at first sight, seem a paradoxical concept. Although acute application of ozone might increase the concentration of lipid hydroperoxides, rapidly buffering by the antioxidant system, repeated stimulation appears to induce an antioxidant response [52]

Conclusion

The results of the present study demonstrate that NO and ROS may be responsible, at least in part, for CP-induced bladder damage. Furthermore, OT may ameliorate bladder damage by scavenging ROS and reducing NO levels.

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