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Protective Effect of Seleno-L-Methionine on Cyclophosphamide-Induced Urinary Bladder Toxicity in Rats

Adnan Ayhanci · Suzan Yaman · Varol Sahinturk · Ruhi Uyar · Gokhan Bayramoglu · Hakan Senturk · Yilmaz Altuner · Sila Appak · Sibel Gunes

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Abstract Cyclophosphamide (CP) is a widely used antineoplastic drug, which could cause toxicity of the normal cells due to its toxic metabolites. Its urotoxicity may cause dose-limiting side effects like hemorrhagic cystitis. Overproduction of reactive oxygen species (ROS) during inflammation is one of the reasons of the urothelial injury. Selenoproteins play crucial roles in regulating ROS and redox status in nearly all tissues; therefore, in this study, the urotoxicity of CP and the possible protective effects of seleno-L-methionine (SLM) on urinary bladder of rats were investigated. Intraperitoneal (i.p.) administration of 50, 100, or 150 mg/kg CP induced cystitis, in a dose-dependent manner, as manifested by marked congestion, edema and extravasation in rat urinary bladder, a marked desquamative damage to the urothelium, severe inflammation in the lamina propria, focal erosions, and polymorphonuclear (PMN) leukocytes associated with occasional lymphocyte infiltration determined by macroscopic and histopathological examination. In rat urinary bladder tissue, a significant decrease in the endogenous antioxidant compound glutathione, and elevation of lipid peroxidation were also noted. Pretreatment with SLM (0.5 or 1 mg/kg) produced a significant decrease in the bladder edema and caused a marked decrease in vascular congestion and hemorrhage and a profound improvement in the histological structure. Moreover, SLM pretreatment decreased lipid peroxide significantly in urinary bladder

A. Ayhanci (✉) · S. Yaman · G. Bayramoglu · H. Senturk · Y. Altuner · S. Gunes
Faculty of Arts and Science, Department of Biology, Meselik Campus, Izmir Osmangazi University, F5 26480 Eskisehir, Turkey
e-mail: aayhanci@ogu.edu.tr

V. Sahinturk
Department of Histology, Faculty of Medicine, Izmir Osmangazi University, Eskisehir, Turkey

R. Uyar
Department of Physiology, Faculty of Medicine, Izmir Osmangazi University, Eskisehir, Turkey

S. Appak
Izmir Institute of Technology Department of Molecular Biology & Genetics,
Biology & Genetics, Izmir Institute of Technology, Eskisehir, Turkey

tissue, and glutathione content was greatly restored. These results suggest that SLM offers protective effects against CP-induced urinary bladder toxicity and could be used as a protective agent against the drug toxicity.

Keywords Cyclophosphamide · Seleno-L-methionine · Urotoxicity · Cytoprotectivity · Rats

Introduction

Cyclophosphamide (CP) is extensively used as an antineoplastic agent for the treatment of various cancers and as an immunosuppressive agent for organ transplantation [1]. CP has been shown to induce severe hemorrhagic cystitis (HC) in laboratory animals [2] and in patients receiving the drug as a part of their treatment [3]. HC is the major dose-limiting side effect of CP [4]. The incidence of this side effect is dose dependent and can be as high as 75% in patients receiving a high intravenous CP dose.

There have been many attempts to prevent CP-induced cystitis with the pretreatments of different drugs, such as zinc or 2-mercaptoethane sulfonate (MESNA). This compound has been shown to reduce, in part, the magnitude of CP-induced cystitis [5]. It is important to elucidate the mechanism of CP-induced HC in order to minimize the toxic and dose-limiting side effects of CP. Elimination the side effects of CP can lead to better tolerance of the drug and a more efficient and comfortable therapy for patients in need of CP treatment [6]. The overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) during inflammation leads to considerable oxidant stress, cellular injury, and necrosis via several mechanisms including peroxidation of membrane lipids, protein denaturation, and DNA damage [7].

It is suggested that CP treatment of rats induces oxidative stress in the urinary bladder and depletion of antioxidant enzyme, and the oxidative stress contributes to neutrophil infiltration into bladder and hence inflammation [6]. Furthermore, Das et al. [8] have reported that CP-induced testicular oxidative stress was manifested by a significant inhibition of peroxidase and catalase enzyme activities and with high levels of malondialdehyde (MDA) and conjugated dienes in the testis.

Numerous studies have shown that CP exposure enhances intracellular ROS production, suggesting that biochemical and physiological disturbances may result from oxidative stress [9]. The antioxidative defense system includes enzymes, such as glutathione peroxidase (GPx), superoxide dismutase, catalase [10], and non-enzyme antioxidants, like carotenoids [11] and selenium [12]. The cellular antioxidant system helps to minimize ROS-induced tissue injury [6]. Thus, the combination of the drug delivery together with potent antioxidant may be the appropriate approach to reduce the side effects of CP [1].

Selenium (Se) is a nutritionally essential trace element with anticarcinogenic properties. Overexpression of Gpx has been reported to block ROS-induced apoptosis in several cell types, suggesting that inhibition of this enzyme is closely related to apoptotic cell death [12]. Limited data from studies in humans suggest that Se supplementation may enhance immunity, including both humoral and cell-mediated responses [13]. It was demonstrated that Se is a highly effective modulator of the therapeutic efficacy and selectivity of anticancer drugs in nude mice bearing human tumor xenografts of colon carcinoma and squamous cell carcinoma of the head and neck. Thus, the use of Se as selective modulator of the therapeutic efficacy of anticancer drugs is new and novel [14]. Therefore, the aim of the present study was to investigate the potential protective effect of seleno-L-methionine (SLM) against CP-induced cystitis in rats.

Materials and Methods

Animals

A total of 84 either sex Sprague–Dawley rats weighing 190–220 g were used for the intraperitoneal injection of CP (Endoxan, Sigma-Aldrich, Taufkirchen, Germany; C0768) and SLM (Sigma, Germany; S3132). Animals were given food and water ad libitium. Local institutional animal care and use committee approved the experimental protocol.

Experimental Design

The rats were randomly divided into the following experimental groups, each including seven animals: groups 1, 2, and 3 treated with 50, 100, or 150 mg/kg CP, respectively [15]. Groups 4 and 5 treated with 0.5 and 1 mg/kg SLM, respectively. Groups 6, 7, or 8 treated with respective CP plus 0.5 mg/kg SLM. Groups 9, 10, or 11 were treated with respective CP plus 1 mg/kg SLM. Group 12 is the control group that only received saline. Animal groups and their treatments are summarized in Table 1.

Tissue Preparation and Histopathology

At the end of the experiment, the rats were weighed. Under the deeper ether anesthesia, the animals were killed at day 7. The bladders were removed intact, evacuated of residual urine, washed with saline, blotted dry on a filter paper, and weighed to determine the presence of edema. The bladders were then cut into two equal pieces from the dome to the bottom. One half was stored at −80°C to measure bladder MDA and glutathione (GSH) contents; the rest was fixed for 24 h in 10% buffered formaldehyde. Tissues were embedded in paraffin and serial cross-sections 4–5-µm thick were taken from each bladder and stained with hematoxylin–eosin. All sections were examined under ×40 objective and scored for edema, hemorrhage, and inflammation on a scale of 0 (normal) to 3 (severe changes). Mucosal ulceration was scored as 0 (normal), 1 (epithelial denuding), 2 (focal ulceration), and 3 (widespread epithelial ulceration) [5–7, 9–16].

Malondialdehyde

MDA content was measured as described by Ohkawa et al. in 1979 [17]. Bladder tissue was homogenized in appropriate buffers and used for the following assays. The mixture consisted of 0.8 ml of sample (1 mg), 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% glacial acetic acid adjusted to pH3.5, and 1.5 ml of 0.8% aqueous solution of 2-thiobarbituric acid. The mixture was made up to 4 ml with distilled water and heated at 95°C for 60 min using a glass ball as condenser. After cooling with tap water, 1 ml distilled water and 5 ml *n*-butanol and pyridine mixture (15:1) were added, and the solution was shaken vigorously. After centrifugation at 2,000×*g* for 10 min, the absorbance of the organic layer was measured at 532 nm. Amount of thiobarbituric reacting substances formed is calculated from standard curve prepared using 1,1′,3,3′ tetramethoxy propane and the values expressed as nanomole MDA per gram of tissue.

Glutathione

Tissue levels of acid-soluble thiols, mainly GSH, were determined colourimetrically at 412 nm. Briefly, 0.5 mL of the previously prepared homogenate was added to

Table 1 Groups of Rats (n=7) and their Treatments

	Days						
	1	2	3	4	5	6	7
Group 1	Saline	Saline	Saline	CP (50 mg/kg)	Saline	Saline	X
Group 2	Saline	Saline	Saline	CP (100 mg/kg)	Saline	Saline	X
Group 3	Saline	Saline	Saline	CP (150 mg/kg)	Saline	Saline	X
Group 4	SLM (0.5 mg/kg)	SLM (0.5 mg/kg)	SLM (0.5 mg/kg)	SLM (0.5 mg/kg)	SLM (0.5 mg/kg)	SLM (0.5 mg/kg)	X
Group 5	SLM (1 mg/kg)	SLM (1 mg/kg)	SLM (1 mg/kg)	SLM (1 mg/kg)	SLM (1 mg/kg)	SLM (1 mg/kg)	X
Group 6	SLM (0.5 mg/kg)	SLM (0.5 mg/kg)	SLM (0.5 mg/kg)	SLM (0.5 mg/kg) + CP (50 mg/kg)	SLM (0.5 mg/kg)	SLM (0.5 mg/kg)	X
Group 7	SLM (0.5 mg/kg)	SLM (0.5 mg/kg)	SLM (0.5 mg/kg)	SLM (0.5 mg/kg) + CP (100 mg/kg)	SLM (0.5 mg/kg)	SLM (0.5 mg/kg)	X
Group 8	SLM (0.5 mg/kg)	SLM (0.5 mg/kg)	SLM (0.5 mg/kg)	SLM (0.5 mg/kg) + CP (150 mg/kg)	SLM (0.5 mg/kg)	SLM (0.5 mg/kg)	X
Group 9	SLM (1 mg/kg)	SLM (1 mg/kg)	SLM (1 mg/kg)	SLM (1 mg/kg) + CP (50 mg/kg)	SLM (1 mg/kg)	SLM (1 mg/kg)	X
Group 10	SLM (1 mg/kg)	SLM (1 mg/kg)	SLM (1 mg/kg)	SLM (1 mg/kg) + CP (100 mg/kg)	SLM (1 mg/kg)	SLM (1 mg/kg)	X
Group 11	SLM (1 mg/kg)	SLM (1 mg/kg)	SLM (1 mg/kg)	SLM (1 mg/kg) + CP (150 mg/kg)	SLM (1 mg/kg)	SLM (1 mg/kg)	X
Group 12	Saline	Saline	Saline	Saline	Saline	Saline	X

CP cyclophosphamide, *SLM* seleno-L-methionine, *X* killed

0.5 mL of 5% trichloroacetic acid, and after centrifugation at 750×*g* for 5 min, the supernatant (200 μL) was added to a tube containing 1,750 μL of 0.1 mol/L potassium phosphate buffer, pH 8, and 50 μL 5,5′-dithiobis-(2-nitrobenzoic acid) reagent. Tubes were mixed, and the yellow color developed was measured against a standard curve of GSH. The protein thiol (protein-SH) content was expressed as micromole per gram of tissue [16].

Statistics

The results were expressed as means±SEM and $p<0.05$ accepted as statistically significant. Statistical analysis was performed using one-way analysis of variance for MDA and GSH levels, body, and bladder weights. Histopathological score points were analyzed first by using the non-parametric Kruskal–Wallis test to discover whether there was any difference between groups. The Tukey honestly significantly different test was then performed to analyze two groups consecutively.

Results

All histological parameters and urinary bladder weights are summarized in Table 2. Three doses of CP (groups 1, 2, and 3) induced acute urinary bladder damage. This was manifested by a significant increase in urinary bladder weight as a ratio of bodyweight, reaching a more than fourfold (exclude group 1) increase with marked edema. Control animals had histologically normal bladders with assigned scores of “0” for all four conditions, edema, hemorrhage, inflammation, and ulceration. Severe histological changes and higher grades of hematuria were observed for animals receiving CP (groups 2 and 3), and severe ulceration and erosion was encountered, as shown in Fig. 1. In addition, histological examination revealed that CP induced extensive inflammation of the lining urothelium, petechial hemorrhage in the lamina propria associated with proteinaceous

Table 2 Comparison of Histological Damage and Bladder/Body Weight (blw/bw) Ratio of Rat Bladders [Median (Min–Max)]

Groups (mg/kg)	Edema	Hemorrhage	Inflammation	Ulceration	Total damage	blw/bw (mg/g)
Control	0	0	0	0	0	0.53 (0.42–0.68)
50 CP	7	0	3	3	13	0.77* (0.67–0.88)
100 CP	17*	5	11	11	42*	2.12* (1.89–2.43)
150 CP	19*	8*	12*	15*	54*	2.39* (1.99–2.77)
0.5 SLM	0	0	0	3	3	0.51 (0.40–0.66)
1 SLM	0	0	0	4	4	0.48 (0.43–0.71)
50 CP +0.5 SLM	0	0	0	0	0	0.56* (0.41–0.70)
100 CP+0.5 SLM	4	1	3	3	11	0.85** (0.68–1.12)
150 CP+0.5 SLM	3	3	4	6	16	0.83** (0.65–1.11)
50 CP+1 SLM	2	0	0	8	10	0.62* (0.47–0.84)
100 CP+1 SLM	7	3	7	8	25	0.72** (0.58–0.98)
150 CP+1 SLM	1	3	0**	5	9	0.67** (0.51–0.83)

*$p<0.05$ compared with control group; **$p<0.05$ compared with CP group

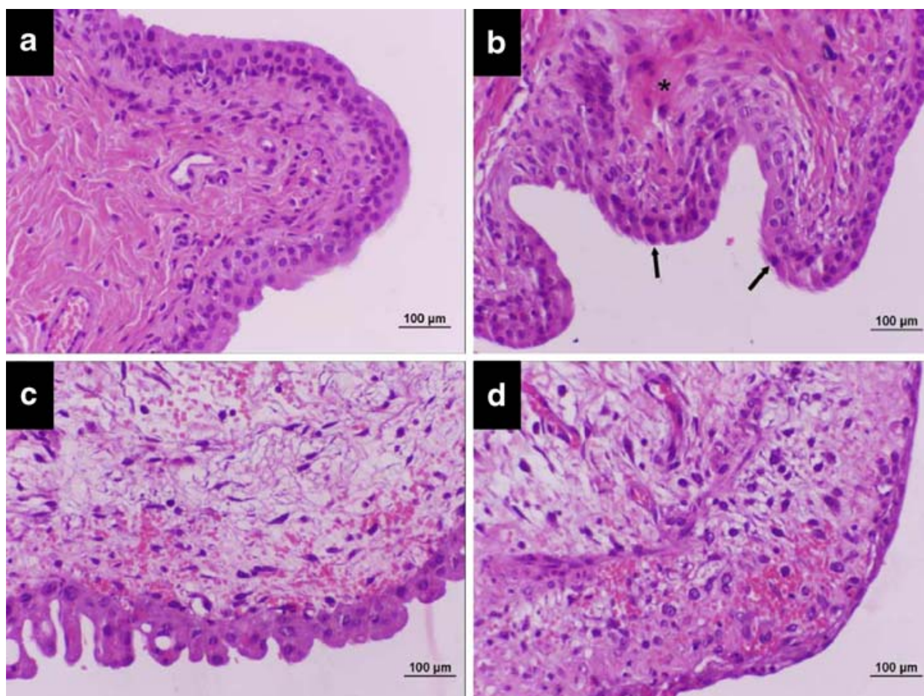

Fig. 1 Representative light micrographs showing bladder histology. **a** Bladder taken from the control rats showing normal histology. **b** A bladder section of a CP 50-treated rat showing edema in the connective tissue and condensed nuclei (*arrow*) in some epithelial cells. **c** A bladder section taken from a CP 100-treated rat showing interruptions in the epithelium. There are hemorrhage and edema in the connective tissue. **d** A bladder section of a CP 150-treated rat showing epithelial disappearance with edema and hemorrhage in the connective tissue

material infiltrating into the lumen and infiltration of erythrocytes, desquamative epithelium as well as PMN leukocytes with focal erosions of the urinary bladder. Pretreatment with SLM (groups 6, 7, 8, 9, 10, and 11) caused a significant decrease in urinary bladder weight (Table 2) and markedly decreased the vascular congestion and hemorrhage and resulted in a profound improvement in the histological structure of the urinary bladder (Figs. 2 and 3). SLM alone caused slight histopathological changes (ulceration) in the normal structure of the urinary bladder (Table 2).

CP injection resulted also in increased MDA levels (two- to fivefold) and decreased GSH levels (by 64%), indicating that oxidative stress was present in the bladders. Pretreatment with SLM ameliorated CP-induced cystitis, as indicated by a significant decrease in lipid peroxides (MDA) and a marked increase of antioxidant (GSH) levels (Figs. 4 and 5).

Discussion

HC is a major therapy-limiting side effect of CP. The main features of HC are urothelial damage, transmural edema, hemorrhage, mucosal ulceration, and epithelial necrosis. These can be demonstrated within 24 h of a single dose [18]. The urotoxicity of CP is thought to be due to the formation of acrolein that damages the urothelium [19, 20]. In accordance

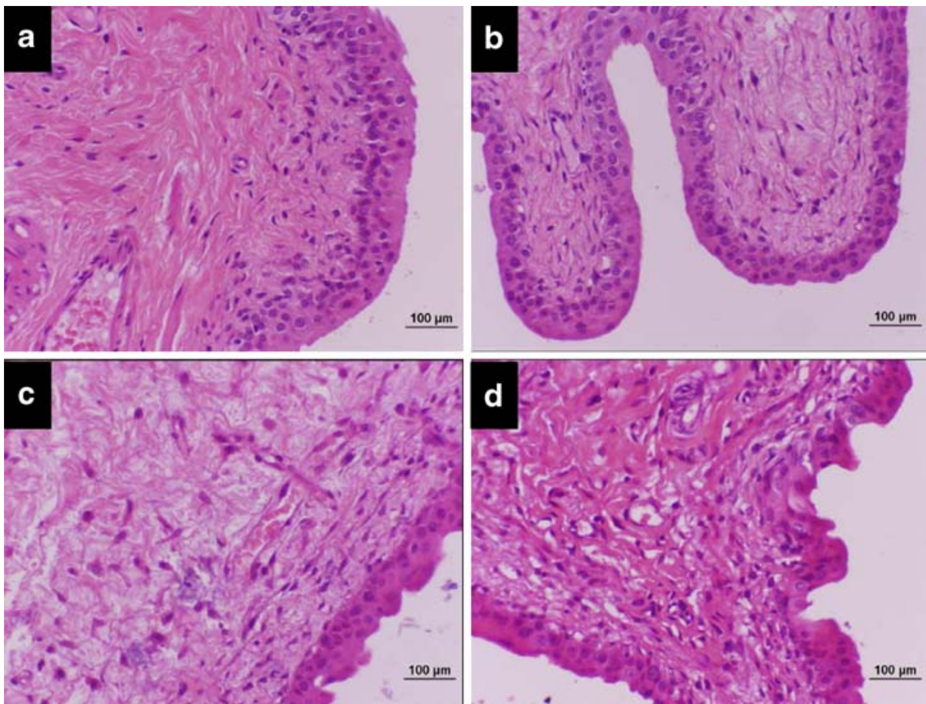

Fig. 2 Representative light micrographs showing bladder histology. Sections showing normal histology from; **a** SLM 0.5-treated, **b** a SLM 0.5-+CP 50-treated rat. **c** Bladder taken from a SLM 0.5-+CP 100-treated rat showing a thin, discontinuous epithelium and edema in the connective tissue. d A bladder section from a SLM 0.5-+CP 150-treated rat showing a partially thin, discontinuous epithelium but no hemorrhage and edema in the connective tissue

with other studies, the damage caused in this study by CP to the structure of the bladders increased as the dose rose. Similarly structural damage was obtained when 100 mg/kg CP was injected to rats [5]. In the present study, treatment with CP produced marked congestion and edema in rat bladder. Histopathological examinations also showed petechial hemorrhage and inflammatory reaction in the lamina propria of the urinary bladder and proteinaceous material in the lumen. PMN leukocytes with occasional lymphocyte infiltration and focal erosions were also observed in urinary bladders from CP-treated rats. These results are consistent with those of previous studies reporting that CP caused excessive protein extravasation, vascular congestion, and edema of the bladder and extensive leukocyte production of NO [21, 22].

HC is now accepted as a non-microbial inflammation, and the pathogenesis of HC may be summarized as cytokine production leading to inducable nitric oxide synthase (iNOS) induction. There is evidence that urinary bladder epithelial cells express reactivity to iNOS in the cytoplasm, leading to peroxynitrite production [22]. Increased NO production is probably responsible for the cystitis because *S*-methylisothiourea (an iNOS-selective inhibitor) almost abolishes CP-induced bladder damage [11]. This improvement is thought to result from a decrease in NO production. In addition, CP produced a decrease in the endogenous antioxidant compound GSH and an elevation of lipid peroxidation in urinary bladder [16, 18–22]. Our findings are in agreement with those of these studies, in which there was a significant depletion of GSH pool and a significant increase in lipid peroxides

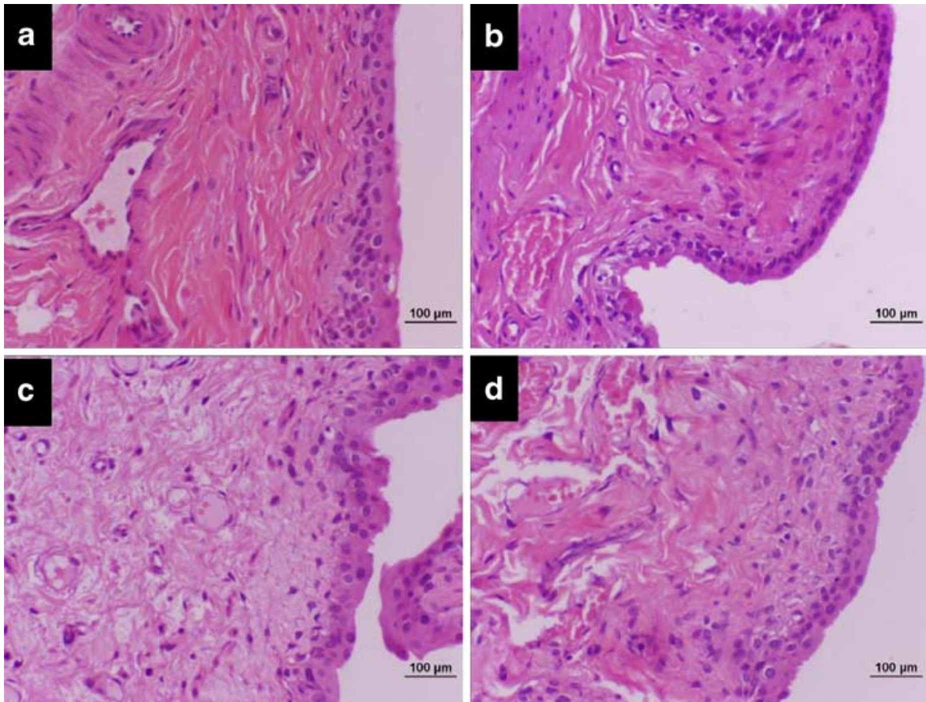

Fig. 3 Representative light micrographs showing bladder histology. A section from **a** SLM 1-treated rat showing a mild ulceration. **b** SLM 1-+CP 50-treated rat showing a mild discontinuous epithelium and a mild edema in the connective tissue. **c** SLM 1-+CP 100-treated rat showing mild changes in the epithelium and edema in the connective tissue. **d** SLM 1-+CP 150-treated rat showing a mild discontinuous epithelium and edema in the connective tissue

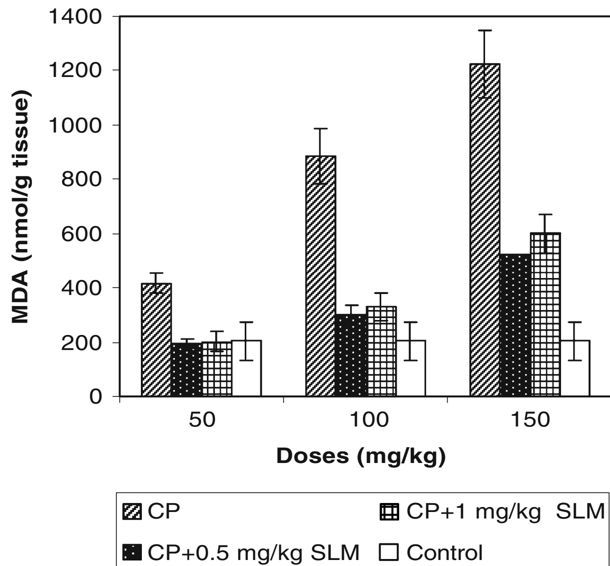

Fig. 4 MDA levels of the bladder. CP administration severely increased MDA in groups 1, 2, and 3 (51%, 77%, and 83%, respectively, $p<0.001$). CP and SLM decreased MDA level in groups 6, 7, and 9 nearly to the level of the control group's ($p>0.05$). In group 8, MDA level was increased by60% compared to the control ($p<0.001$), whereas decreased by 58% compared to group 3 ($p<0.001$). In group 10, MDA level was increased by 36% compared to the control ($p<0.01$) and found to be decreased by64% compared to group 2 ($p<0.001$). In group 11, MDA level was increased by66% compared to the control ($p<0.001$) and found to be decreased by 51% compared to the third group ($p<0.001$)

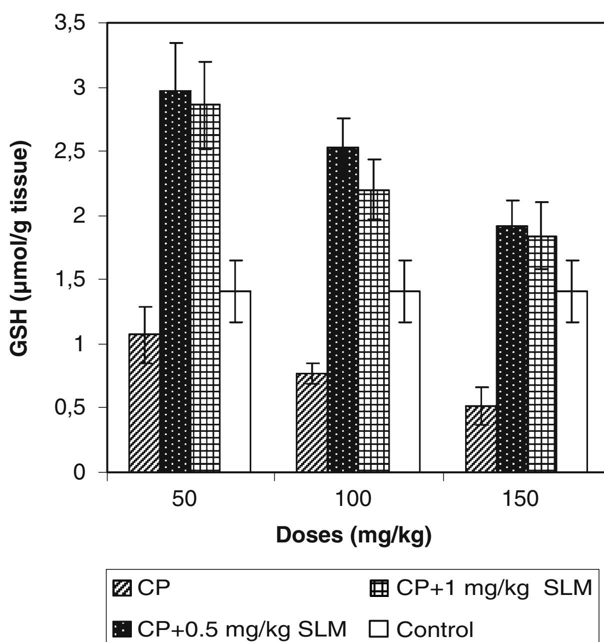

Fig. 5 GSH levels of the bladder. GSH levels were found to be decreased dose dependently only in the first three groups that received CP alone compared to the control. This reduction (25%) was not statistically significant in group 1 ($p>0.05$); in groups 2 (46%) and 3 (64%), the reduction was statistically significant ($p<0.001$). When administrated with CP, SLM raised the antioxidant GSH levels in group 6 by 53%, group 7 by 45%, group 9 by 51%, and in group 10 by 36%, and these were found to be statistically highly significant ($p<0.001$); in group 8, it raised the GSH level significantly (26%, $p<0.01$). The increase in the antioxidant GSH levels in group 11 (% 23) was not found to be statistically significant ($p>0.05$)

(MDA) in urinary bladder tissues after CP injection. Similarly, it was reported that CP produced a marked decrease in urinary bladder GSH content [23]. GSH is the major cellular sulphydryl compound that serves as both a nucleophile and an effective reductant by interacting with numerous electrophilic and oxidizing compounds. It can act as a non-enzymic antioxidant by direct interaction of –SH group with ROS, or it can be involved in the enzymatic detoxification reaction for ROS, as a cofactor or coenzyme. The depletion of GSH content may be attributed to the direct conjugation of CP and its metabolites with free or protein bound –SH groups [24], thereby interfering with the antioxidant functions. Decreased GSH content can explain the additional decreased concentration of vitamin C, which enters the cell mainly in the oxidized form where it is reduced by GSH [25]. Besides other protective drugs, recently, amifostine and glutathione were reported to prevent acrolein-induced HC in mouse bladder; however, the efficacy of these agents in humans has yet to be determined [26].

Previous studies have shown that CP toxicity is associated with oxidative cell damage [27]; thus, therapeutic strategies aim to limit free radical-mediated urinary bladder injury [28].

Se is recognized to have a capacity for conferring tolerance to the toxic manifestations of various heavy metal exposures, including cadmium, mercury, lead, and arsenic [29, 30] in addition to role as an anticancer agent [31]. Moreover, Se has protective effects against mercury toxicity in rat kidney and Se also used to diminish the toxic effects of the cadmium on the antioxidant enzyme system, which in turn affects the membranes structures such as mitochondria and endoplasmic reticulum. On the other hand, Se deficiency reducing GPx activity has been reported in diet of glomerular disease and in diet of tubular epithelium in normal rats [32]. Concurrent or prior injections of Se were found to protect against many of the acute effects of cadmium, e.g., testicular necrosis, placental hemorrhage, teratogenecity, and damage to the lactating mammary glands [14–16, 18–31, 33].

Olas and Wachowicz [34] has reported that the administration of sodium selenite appears to reduce cisplatin toxicity without inhibiting the antitumor activity of cisplatin. Weijl et al. [35] have found that supplementation with a higher dose of Se in combination with other antioxidants (vitamins C and E) could correlate with cisplatin-induced autotoxicity and nefrotoxicity. While the deprivation of Se can reduce the protection against oxidative stress and impair immunocompetence, certain cancer cells appear to have acquired a selective survival advantage that is apparent under conditions of Se deficiency and oxidative stress [36].

The evidences obtained from our study indicate that pretreatment with SLM ameliorates CP-induced cystitis because it leads to a reduction in the macroscopic changes induced by CP, such as hemorrhage and edema, as well as a profound improvement in the histological structure and shape. Both SLM doses that we used improved bladder morphology in a dose dependent manner. SLM also offers marked protection against extensive CP-induced inflammation. Furthermore, pretreatment with SLM ameliorated CP-induced cystitis, as indicated by a significant decrease in MDA and a marked increased of antioxidant GSH levels in bladder tissue. All histopathological examinations and biochemical measurements suggest that Se may have a role in two stages, namely, prevention and interception, of defense against ROS and RNS production.

In essence, the results of the present study suggest that CP-induced cystitis is related to oxidative stress and ROS formation owing to depletion of GSH and/or inflammation. Se can also protect the structure and function of the urinary bladder against CP-induced oxidative stress. Our findings suggest that, at convenient concentration, selenium could be a potentially effective drug in the treatment of CP-induced cystitis. We believe that additional experimentation should be performed to explore the underlying mechanism of Se protection against CP toxicity.

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