Supportive Care in Cancer (2019) 27:4273–4281
https://doi.org/10.1007/s00520-019-04731-2

ORIGINAL ARTICLE

Uroprotective effect of pantoprazole against cyclophosphamide-induced cystitis in mice

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Received: 19 November 2018 / Accepted: 5 March 2019 / Published online: 14 March 2019

Abstract

Purpose The aim of the present study was to evaluate the potential uroprotective effect of pantoprazole (PPZ) in a mouse model of cyclophosphamide (CP)-induced hemorrhagic cystitis (HC) due to its antioxidant and anti-inflammatory properties.

Methods Balb/c mice received a single intraperitoneal (i.p.) injection of CP (300 mg/kg) to induce HC. PPZ (20, 50, and 100 mg/kg/day;i.p.) was administered for 3 consecutive days before the induction of HC. Mesna (30 mg/kg;i.p.) was administered 20 min before, 4 and 8 h after CP injection to compare the protective effects of PPZ. After 24 h of HC induction, the bladders were removed for functional studies, biochemical analyses, and histopathological examination.

Results In vitro contractility studies demonstrated that CP-induced HC decreased the responsiveness of detrusor muscle strips to acetylcholine (ACh), which was reversed by PPZ pretreatment at all doses tested. However, mesna treatment was not able to improve responsiveness to ACh. Biochemical analyses showed that CP caused significant elevation of malondialdehyde (MDA), reduction of total glutathione (GSH), and increment of proinflammatory cytokine tumor necrosis factor-alpha (TNF-α) level, which were measured in bladder homogenates. PPZ pretreatment at three doses found to be effective in reducing the CP-induced elevation of MDA and TNF-α levels. The highest dose of PPZ (100 mg/kg) caused a significant increase in GSH level. CP induced severe HC with marked bladder edema and histological disturbances which were partially abolished by PPZ pretreatment.

Conclusions Our results indicate that PPZ pretreatment could attenuate CP-induced HC by interfering with oxidative stress and modulating proinflammatory cytokines.

Keywords Antioxidant · Cyclophosphamide · Detrusor contractility · Hemorrhagic cystitis · Pantoprazole · Proinflammatory cytokine

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Introduction

Cyclophosphamide (CP) is an alkylating antineoplastic drug that has been proven to be effective in the treatment of various malignancies and non-malignant disease states such as some immune-related disorders [1, 2]. The clinical use of CP is limited due to toxic side effects such as hemorrhagic cystitis (HC), alopecia, hematopoietic depression, gonadal dysfunction, nausea, vomiting, fulminant cardiac toxicity, and gastrointestinal, renal, and lung toxicities [3, 4]. HC is a major dose-limiting serious side effect of CP. The incidence of HC ranges from 2 to 40% related to dosage regimen and can be as high as 75% in patients receiving a high intravenous dose of CP treatment [5, 6]. Acrolein is the causative urotoxic metabolite of CP leading to HC characterized by urothelial damage, subepithelial edema, hemorrhage, leukocyte infiltration, and necrosis [7, 8]. Mesna (2-mercaptoethane sulfonate sodium) is the only approved drug to protect against CP-induced HC by detoxifying acrolein. However, some cases of HC demonstrating poorer response and mesna-related side effects are still clinically encountered [6, 9]. Numerous studies have reported that reactive oxygen species (ROS) and inflammatory mediators are involved in the pathogenesis of CP-induced HC. Therefore, antioxidants and anti-inflammatory agents have attracted great interest as promising therapeutic candidates to treat CP-induced HC for improving tolerability of CP treatment [10, 11].

Proton pump inhibitors (PPIs) are substituted benzimidazole derivates that suppress gastric acid secretion by inhibiting H^+/K-ATPase enzyme in the gastric parietal cell [12]. Clinically approved PPIs including pantoprazole (PPZ) are widely used for the treatment of acid-peptic disorders such as gastric ulcer, duodenal ulcer, and gastro-esophageal reflux disease [13]. Recent studies have shown that PPIs have also antioxidant and anti-inflammatory properties in vitro and in vivo unrelated to the inhibition of gastric acid production. It is well established that PPIs are able to increase glutathione (GSH) levels and they have also high ROS scavenging capacity that improves mucosal function. Moreover, PPIs were found to decrease the levels of proinflammatory cytokines indicated in several experimental inflammatory conditions [14].

Therefore, in the present study, we aimed to evaluate the potential protective effect of PPZ against CP-induced cystitis in mice due to its antioxidant and anti-inflammatory properties.

Materials and methods

Drugs and chemicals

Cyclophosphamide (CP) (Endoxan® 500 mg vial, Eczacıbaşı Baxter, Turkey) and Mesna (Uromitexan® 400 mg, Eczacıbaşı Baxter, Turkey) were used. Acetylcholine (ACh) and pantoprazole (PPZ) were purchased from Sigma (St. Louis, MO, USA). In addition, tumor necrosis factor-alpha (TNF-α) (R&D Systems, USA) and GSH (Cayman Chemical, USA) commercial kits were used for biochemical analysis. CP, mesna, and PPZ were dissolved or diluted in 0.9% sterile saline.

Animals

Adult male Balb/c mice (6–8 week-old) weighing 25–35 g were used in the study. All animals were housed in plastic cages maintained under temperature–humidity–controlled conditions (22 ± 2 °C and 60 ± 5%, respectively) with a regular 12-h light–dark cycle and were allowed free access to standard commercial pellet diet and water. The experimental protocol was approved by the Local Ethics Committee of Faculty of Medicine (protocol approval number: 2016/29) as Institutional Review Board. All animal studies were performed according to the Guide for the Care and Use of Laboratory Animals.

Experimental design

Animals were divided randomly into 6 groups of 21 animals each (7 animals were used for the assessment of detrusor contractility, 7 animals were used for biochemical analysis, and 7 animals were used for histopathological examination per group). Animals were treated as follows.

Control group Mice were pretreated with saline (the vehicle of PPZ) once a day for 3 consecutive days. On the third day 30 min after the last dose of saline, saline was received as the vehicle of CP.

CP group Mice were pretreated with saline once a day for 3 consecutive days. On the third day 30 min after the last dose of saline, CP (300 mg/kg) was received to induce HC.

CP+Mesna group Mice were treated with Mesna (30 mg/kg) 20 min before and 4 and 8 h after cystitis induction with a single dose of CP (300 mg/kg).

CP+PPZ_{20} group Mice were pretreated with PPZ (20 mg/kg) once a day for 3 consecutive days. On the third day 30 min after the last dose of PPZ, CP (300 mg/kg) was received to induce HC.

CP+PPZ_{50} group Mice were pretreated with PPZ (50 mg/kg) once a day for 3 consecutive days. On the third day 30 min after the last dose of PPZ, CP (300 mg/kg) was received to induce HC.

CP+PPZ$_{100}$ group Mice were pretreated with PPZ (100 mg/kg) once a day for 3 consecutive days. On the third day 30 min after the last dose of PPZ, CP (300 mg/kg) was received to induce HC.

All drugs were injected intraperitoneally. Doses of drugs and protocol were selected depending on previous studies with some modifications [15–17]. Animals were killed by cervical dislocation 24 h after the administration of CP or saline (on the fourth day of the experiment). Urinary bladders were quickly dissected out and then a part of them was frozen immediately in liquid nitrogen and stored at − 80 °C until use for biochemical analyses. The other part of the bladder was used to prepare detrusor strips to evaluate contractile function in vitro and histological assessment.

Contractility studies

The bladders were immediately removed and detrusor smooth muscle strips (3–5-mm long, 2–3-mm wide) were prepared from each animal. Strips were mounted between two steel hooks in a 30 mL isolated tissue chamber containing gassed (95% O_2 and 5% CO_2) Krebs–Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM NaH_2PO_4, 1.3 mM $MgSO_4$ 1.3, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11 mM glucose) at 37 °C. Under a resting optimal tension of 1 g, strips were allowed to equilibrate for 1 h before experiment and the solution was changed every 20 min. Isometric tension was recorded an isometric force displacement transducer (MAY FDT10A) connected to an acquisition system (BIOPAC MP35). After equilibration period, the strips were contracted by KCl (60 mM) to determine tissue viability. Cumulative concentration-response curves to the muscarinic receptor agonist ACh (10^{-8}–10^{-3} M) were obtained. Then, strips were washed with Krebs–Henseleit solution and after an equilibration period, cumulative concentration-response curves to ACh (10^{-8}–10^{-3} M) were re-obtained. Contractile response was calculated by taking average of maximum tension for each concentration of ACh and normalized to the wet weight of the respective strips. The maximal contractile response was expressed as milligram tension per milligram strip weight.

Biochemical analyses

Preparation of bladder homogenates

Each frozen bladder tissue was homogenized in 5–10 mL cold phosphate buffer saline (PBS, pH 6–7) containing 1 mM EDTA per gram tissue. Then the samples were centrifuged at 4 °C, 10.000×*g* for 15 min. The supernatants which were used for GSH assay were deproteinated according to the manufacturer's instructions at commercial available kit (GSH assay kit, Cayman Chemical, USA). All samples stored at − 20 °C until biochemical analysis.

Determination of malondialdehyde (MDA) levels in urinary bladder homogenates

Tissue levels of peroxides were determined as thiobarbituric acid reactive substances (TBARS) described by Stocks and Dormandy [18]. In the protocol, 1 mL of 15% TCA in 0.25 M HCl was added to 10% tissue homogenate and centrifuged at 2000 rpm for 15 min. 1.2 mL of supernatants were mixed with 0.6 mL 37% TBA in 0.25 M HCl and held in a boiling water bath for 15 min after cooling under the tape water. The absorbance of supernatant was measured spectrophotometrically at 532 nm. TBARS levels were calculated using 1,1,3,3-tetraethoxypropane as the standard and expressed as nanomole MDA per gram protein.

Determination of total GSH content in urinary bladder homogenates

Tissue levels of acid-soluble thiols, mainly GSH, were determined colorimetrically at 412 nm using a commercial available kit (GSH assay kit, Cayman Chemical, USA). The assay was performed according to the manufacturer's instructions and the protein thiol content was expressed as micromole per gram tissue.

Determination of TNF-α levels in urinary bladder homogenates

Level of TNF-α was determined by ELISA technique in urinary bladder homogenate using a commercial available kit (R&D system, USA) in accordance with the manufacturer's instructions and TNF-α content was expressed as picogram per milliliter.

Histopathological analyses

The bladder tissues were fixed with 10% formaldehyde, dehydrated through graded alcohols, and then embedded in paraffin. Five-micrometer sections were cut from paraffin blocks using a rotary microtome (RM 2255; Leica Instruments, Nussloch, Germany) and stained with hemotoxylin and eosin (H&E). Slides were observed by a light microscope (Olympus BX51; Olympus Co., Tokyo, Japan) fitted with a digital camera (Olympus DP 71 Olympus Co., Tokyo, Japan). Ten fields of each slide were scored for mucosal edema, leukocyte infiltration, hemorrhage, and mucosal abrasion by the histologist who was blinded to the treatments [19].

Edema was evaluated under × 200 magnification. No edema was scored as 0, minimal edema (no change in connective tissue thickness) as 1, moderate edema (connective tissue thickness increased by < 2-fold) as 2, and severe edema connective tissue thickness increased by (> 2-fold) as 3.

Leukocyte infiltration was evaluated in mucosa under × 400 magnification. No extravascular leukocytes were scored as 0, > 20 leukocytes as 1, 20–45 leukocytes as 2, and > 45 leukocytes as 3.

Mucosal hemorrhage and mucosal abrasion were evaluated under ×100 magnification. The presence of hemorrhage and mucosal abrasion was scored as 1, and no change was scored as 0.

The total score of all fields of view for mucosal edema, leukocyte infiltration, mucosal hemorrhage, and mucosal abrasion divided by the maximum possible score and then multiplied by 100. In addition for edema evaluation, bladders were weighed and relative weight of urinary bladder was determined by calculating the ratio of wet bladder weight (milligram) to body weight (gram) of mice.

Statistical analysis

Data are expressed as the mean + standard error (SEM) or mean + standard deviation (SD). Statistical analysis was performed using SPSS software (version 22; IBM SPSS Statistics, Armonk, NY, USA) and GraphPad Prism (Version 5.01; Graphpad Software, San Diego, CA, USA). The differences between groups were compared with ANOVA, followed by Fisher's least significant difference (LSD) test or Bonferroni's test for parametric data. The Kruskal–Wallis test followed by Dunn's multiple comparison test was used for nonparametric data. $p < 0.05$ was considered to be statistically significant.

Results

PPZ pretreatment improves contractile responses to ACh in the detrusor strips

ACh (10^{-8}–10^{-3} M) produced concentration-dependent contractile responses in the detrusor strips of all groups (Fig. 1a), but the maximal contractile responses to ACh (10^{-8}–10^{-3} M) were significantly decreased in CP group compared with the control group (116.52 ± 12.07 and 329.64 ± 27.54 mg tension/mg tissue, respectively; Fig. 1 b). Mesna treatment failed to prevent the decrease in the contractile response to ACh compared with the CP group. However, pretreatment with PPZ at all doses caused a significant increase in ACh-induced contractions compared with the CP group. There were no significant differences among PPZ-treated groups.

PPZ pretreatment decreases lipid peroxides in urinary bladder homogenates

CP treatment caused a significant increase in lipid peroxides, measured as MDA, in urinary bladder homogenates. However, treatment with mesna and PPZ significantly reduced MDA levels compared with the CP group. Furthermore, PPZ at all doses was found to be more effective in reducing the CP-induced increment of MDA levels than mesna. MDA levels were also significantly lower in CP+PPZ_{100} group compared to the CP+PPZ_{20} and CP+PPZ_{50} groups (Fig. 2).

PPZ pretreatment attenuates CP-induced reduction of GSH content in urinary bladder homogenates

A significant decrease was observed in GSH content of CP group compared with the control group. Although lower doses of PPZ had no effect on GSH content, the highest dose (100 mg kg^{-1}) of PPZ caused a significant increase compared with CP group (Fig. 3).

PPZ pretreatment reduces CP-induced release of TNF-α levels in urinary bladder homogenates

CP group showed a marked increase in the level of TNF-α as compared with control group. Treatment with mesna and PPZ at all doses tested caused a significant decrease in the TNF-α level. There were no significant differences among mesna and PPZ-treated groups (Fig. 4).

PPZ pretreatment reduces CP-induced damage to bladder tissues

Histopathological analyses revealed that a single dose of CP induced HC and control group bladders showed normal architecture. CP group showed significant damage with mucosal edema, leukocyte infiltration, hemorrhage, and mucosal abrasion. The severity of some histopathological changes was found to be attenuated in PPZ-treated groups. In mesna and PPZ-treated groups, mucosal edema was found to be significantly reduced compared with CP group. CP-induced HC caused leukocyte infiltration, which was markedly reduced in CP+Mesna, CP+PPZ_{50} and CP+PPZ_{100} groups. Mesna and PPZ treatment caused a reduction in mucosal hemorrhage, which was not significant compared with the CP group. CP+Mesna group showed significantly decreased mucosal abrasion compared with CP group. Although PPZ treatment also caused a decrease in mucosal abrasion, it was not statistically significant compared with CP group. The relative bladder weight as a marker of edema was significantly higher in CP group compared with control group. PPZ and Mesna treatment caused a significant decrease (Fig. 5(a–f), Table 1).

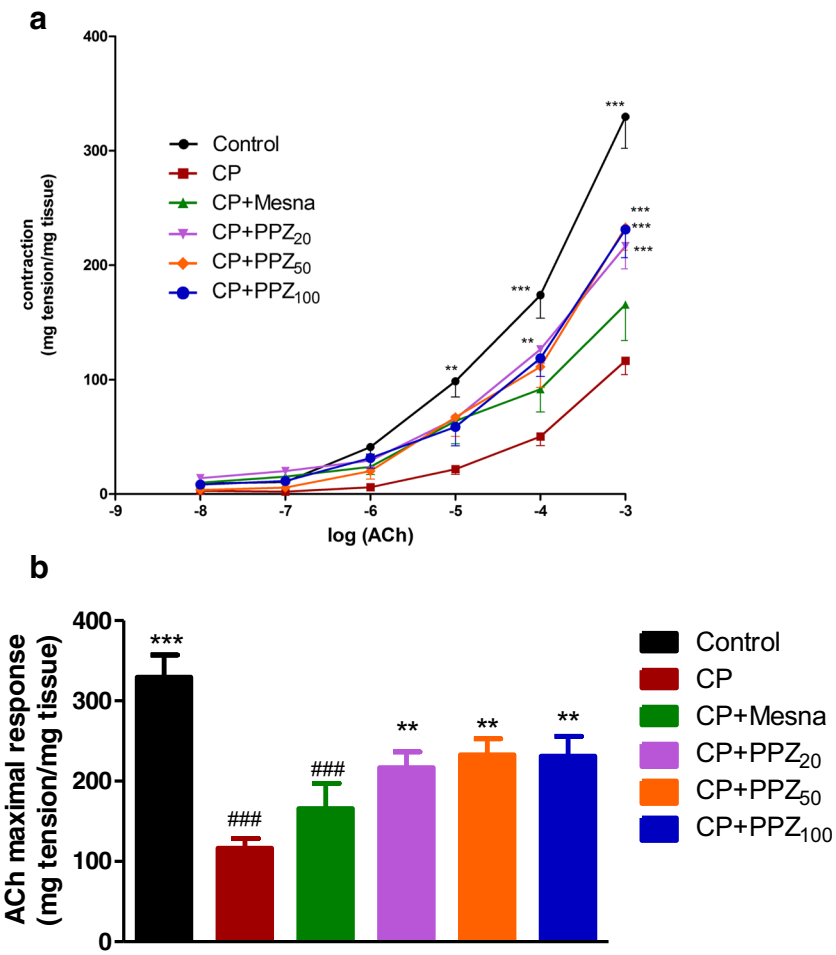

Fig. 1 Cumulative concentration-response curves for ACh (10^{-8}–10^{-3} M) (**a**) and maximal contractile responses to ACh in the detrusor strips of all groups (**b**). Data are expressed as mean ± SEM ($n = 5$–6). $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the CP group, $^{###}p < 0.001$ compared with the control group

Discussion

To our knowledge, this study is the first to report that PPZ pretreatment significantly attenuated CP-induced bladder damage due to its antioxidant and anti-inflammatory effect evidenced by decrease in MDA and TNF-α levels and increase in total GSH content in urinary bladders.

CP, an alkylating cytotoxic drug, has been associated with severe HC. Exact mechanisms of CP-induced HC still remain unclear; however, previous studies have demonstrated that oxidative stress and inflammation play pivotal roles in the urotoxicity of CP. CP-induced urotoxicity is mediated by the production of acrolein, a hepatic microsome-mediated metabolite excreted via urine [7, 20]. The accumulation of the acrolein in the bladder causes urothelium damage by activating ROS production and inflammatory response that nitric oxide (NO) and cytokines such as TNF-α and IL-1β are involved in the processes as crucial mediators [21, 22]. Mesna is an effective uroprotective agent against CP-induced HC by

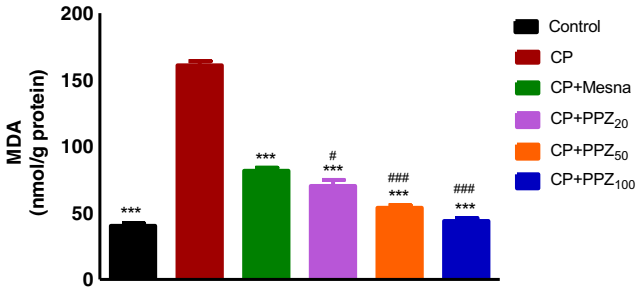

Fig. 2 MDA levels in the urinary bladder homogenates of all groups. Data are expressed as mean ± SEM ($n = 5$). $^{***}p < 0.001$ compared with the CP group; $^{#}p < 0.05$, $^{###}p < 0.001$ compared with the CP+Mesna group

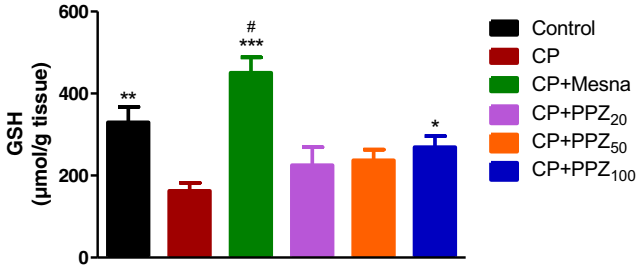

Fig. 3 GSH content in the urinary bladder homogenates of all groups. Data are expressed as mean ± SEM ($n = 5$). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the CP group; $^{#}p < 0.001$ compared with PPZ-treated groups

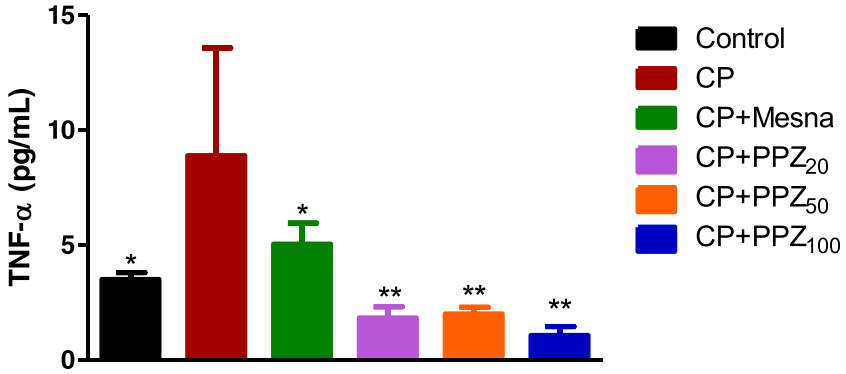

Fig. 4 TNF-α levels in the urinary bladder homogenates of all groups. Data are expressed as mean ± SEM ($n = 5$). *$p < 0.05$, **$p < 0.01$ compared with the CP group

conjugating with acrolein to detoxify. However, mesna is not able to prevent the induction of HC completely and it is also associated with allergic reactions [9, 23]. Therefore, there is an urgent clinical need for novel therapeutics to overcome CP-induced HC.

In the present study, we demonstrated that PPZ, one of PPIs, has uroprotective effect in a mouse model of CP-induced HC by ameliorating oxidative stress and inflammation. Moreover, PPZ improved CP-induced detrusor dysfunction.

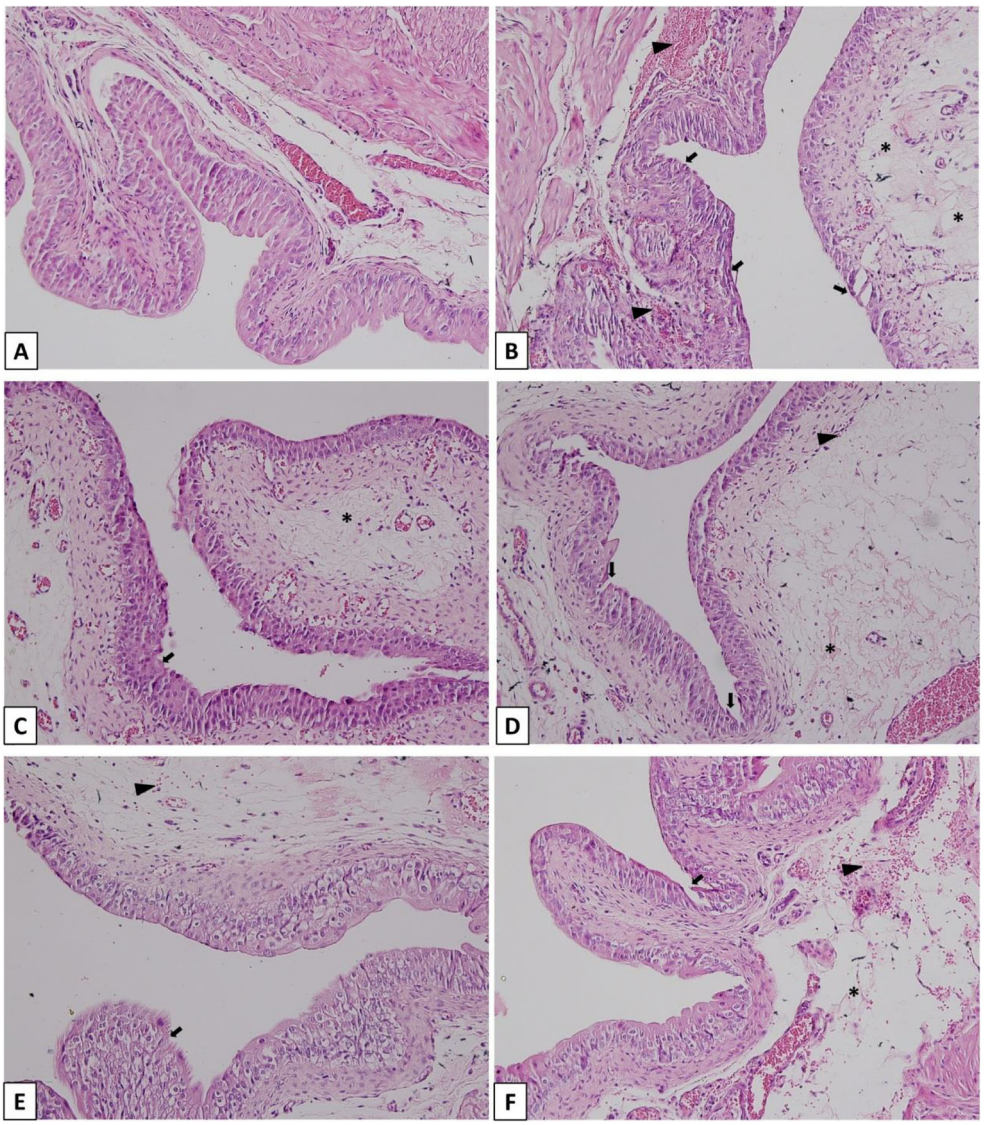

Fig. 5 Representative photographs of bladder sections obtained from all groups. Control group (**a**) showing normal histoarchitecture of bladder with intact epitelium and regular lamina propria. CP group (**b**) showing severe mucosal abrasion (→), marked hemorrhage (▲) and edema **(*)**. CP+Mesna group (**c**) showing significantly decreased edema **(*)** and mucosal abrasion (→). CP+ PPZ_{20} (**d**), CP+ PPZ_{50} (**e**), and CP+ PPZ_{100} (**f**) groups showing decreased the severity of mucosal abrasion (→), edema **(*),** and hemorrhage (▲) (H&E; X200)

Table 1 Histological changes in the urinary bladders and relative bladder weights of groups.

Groups	Histological parameters[a]				Relative bladder weight[b] (mg/g body weight)
	Mucosal edema	Leukocyte infiltration	Mucosal hemorrhage	Mucosal abrasion	
Control	5.00±1.92***	6.66±2.72***	15.00±5.77**	22.50±12.58**	1.50±0.20***
CP	81.66±17.53	75.83±18.54	75.00±19.15	82.50±9.57	3.25±0.22
CP+Mesna	25.00±5.06***	33.89±22.84**	48.33±14.72	38.33±18.35*	1.66±0.12***
CP+PPZ_{20}	49.44±16.79**	50.55±21.75	68.33±23.17	68.33±13.29	2.07±0.17***
CP+PPZ_{50}	34.44±16.42***	32.22±16.28**	55.00±20.74	51.66±19.41	1.75±0.13***
CP+PPZ_{100}	41.11±12.59***	42.22±15.73**	61.66±25.63	58.33±27.87	2.29±0.14***

[a] Data are expressed as mean ± SD ($n = 5–6$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with the CP group

[b] Data are expressed as mean ± SEM ($n = 5–6$). ***$p < 0.001$ compared with the CP group

ACh, the main contractile factor releasing from parasympathetic nerve fibers, produces detrusor smooth muscle contractions via muscarinic receptor activations. M2 receptor subtype is the predominant muscarinic receptor subtype; however, M3 receptor subtype is the most important to mediate cholinergic contractile response [24]. Many studies illustrated that the expression and functional roles of muscarinic receptors are altered by CP in the urinary bladder [25, 26]. Sugino et al. also revealed that mRNA expressions of M2 and M3 receptors increased in the urothelial cells and decreased in detrusor muscles in CP-induced chronic cystitis in rats [27]. In agreement with previous studies [28, 29], we found that in vitro detrusor contractions by muscarinic receptor agonist ACh were reduced in CP-treated mice. Mesna failed to prevent the reduction in the ACh-induced cholinergic contractions. However, PPZ treatments at all doses tested were found to be effective in ameliorating CP-induced reduction in the cholinergic contractions of detrusor strips, suggesting protective effect of PPZ on smooth muscle damage due to CP.

MDA is an end-product of lipid peroxidation, which is a well-established mechanism of cellular injury resulting from oxidative damage of membrane [30]. MDA is widely used as an indicator of oxidative stress and previous studies reported that CP treatment leads to an increase in tissue or blood MDA levels [16, 31]. Our results showed that the administration of CP at 300 mg kg^{-1} caused a marked increase in the MDA level of the urinary bladder. Mesna treatment decreased the MDA levels significantly; however, MDA levels were found to be significantly lower in PPZ-treated groups at all doses compared with CP group and CP+Mesna group. Antioxidant effects of PPIs have been considered one of the mechanisms mediating their gastroprotective actions [14]. Fornai et al. showed that treatment with PPZ (15 μmol/kg/day) for 3 or 7 days caused a significant reduction in the enhancement of mucosal MDA levels in indomethacin-induced gastric mucosal damage model [32]. They also revealed that lansoprazole at 90 μmol/kg markedly attenuated mucosal MDA content in a non-steroidal anti-inflammatory drug (NSAID)-induced gastric injury [33]. Biswas et al. found that omeprazole can block membrane lipid peroxidation and protein oxidation in restraint cold stress-induced gastric ulceration model [34]. Antioxidant properties of PPIs such as lansoprazole and PPZ were attributed to the sulphoxide moiety of the molecules [35].

GSH is the major cellular sulfhydryl compound that serves as a potent endogenous antioxidant. In our study, CP treatment caused a significant decrease in the GSH content of urinary bladders, which is consistent with previous studies [16, 36]. Our study demonstrated that pretreatment with PPZ at 100 mg kg^{-1} increased significantly the GSH content of urinary bladders compared with CP group. Fornai at al. reported that PPZ at 60 μmol/kg increased the mucosal content of GSH in NSAID-induced gastric mucosal damage model [37]. Pastoris et al. also found that esomeprazole (10–60 μmol/kg) dose-dependently reversed the reduction of GSH levels in the gastric mucosa of rats treated with indomethacin [38].

Recent studies have shown that proinflammatory cytokines such as TNF-α and IL-1β play a role in the pathogenesis of CP-induced HC [20, 21]. In our study, CP administration elevated TNF-α level in accordance with previous studies [39, 40]. A significant decrease was observed in mesna as well as PPZ-treated groups.

CP is a prodrug that undergoes hepatic metabolism by cytochrome P-450 enzymes to form acrolein, which is highly toxic metabolite leading to oxidative and inflammatory processes in the pathogenesis of HC [2]. Cytochrome P450 (CYP) enzymes such as CYP2D6, CYP2C19, and CYP2B6 were mainly involved in CP metabolism [41]. PPIs have been reported to have potential for significant drug interactions by elevating intragastric pH and interacting with adenosine triphosphate-dependent P-glycoprotein or with the cytochrome P450 enzyme systems, especially CYP2C19 and CYP3A4 [42]. Omeprazole which has high affinity for CYP2C19 and CYP3A4 might cause clinically significant drug interactions; however, PPZ appears to have lower potential for interactions [43]. Therefore, uroprotective effect of

PPZ seems to be unrelated to the reduction of acrolein formation by inhibiting CP metabolism.

In the present study, we did not investigate the effect of PPZ treatment on the antitumor activity of CP. There are many studies demonstrating PPIs will be employed in novel anticancer strategies by acting as chemosensitizers and direct antitumor effect [44]. Therefore, PPIs might be a promising adjuvant which could attenuate chemotherapy-induced HC and also enhance the efficacy of chemotherapy. Further studies are needed to determine whether there is an interaction between PPZ and CP, resulting in the changes on the antitumor activity of CP.

In conclusion, our study is the first to demonstrate PPZ exerts protective effect in the CP-induced urotoxicity by possibly involving mechanisms related to its antioxidant and anti-inflammatory activities. Our results also provide an evidence indicating that PPZ might be attractive therapeutic for the treatment of chemotherapy-induced bladder damage and dysfunction.

Funding information This study was supported by a grant from the Scientific Research Project Coordination Unit of Karadeniz Technical University (Project no. THD-2018-7356).

Compliance with ethical standards

The experimental protocol was approved by the Local Ethics Committee of Faculty of Medicine (protocol approval number: 2016/29) as Institutional Review Board. All animal studies were performed according to the Guide for the Care and Use of Laboratory Animals.

Conflict of interest The authors declare that they have no conflict of interest.

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