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Dimethyl Fumarate Attenuates Cyclophosphamide-Induced Bladder Damage and Enhances Cytotoxic Activity Against SH-SY5Y Cells

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ABSTRACT

Cylophosphamide (CP)-induced acute cystitis is a debilitating bladder dysfunction commonly observed in cancer patients, primarily resulting from oxidative damage and inflammation in the bladder tissue. Dimethyl fumarate (DMF) is a fumaric acid ester approved for the treatment of multiple sclerosis due to its antioxidant and anti-inflammatory properties. Thus, we aimed to investigate the multiple effects of DMF, involving both its potential synergistic effect with CP on the SH-SY5Y cells and its uroprotective effect on CP-induced acute cystitis. Female Balb/c mice were orally administrated DMF (100 or 300 mg/kg/day) for five consecutive days before a single intraperitoneal (i.p) dose of CP. Mesna was administered 20 min before and at 4 h, 8 h after CP application. Following 24 h of CP injection, bladders were removed for functional, biochemical analysis and evaluation of vesical vascular permeability. SH-SY5Y cell viability was assayed by MTT test. CP markedly decreased carbachol-induced contraction of detrusor strips ($p < 0.01$), which was prevented by the high-dose DMF treatment ($p < 0.05$). Evans blue dye extravasation was greatly increased in the bladders of cystitis group ($p < 0.001$), which was significantly decreased in DMF-treated mice with cystitis ($p < 0.01$). Total GSH content was decreased ($p < 0.01$) whereas TNF-α level was increased ($p < 0.05$) in the bladders of cystitis group. High-dose DMF-treated mice showed an increment in total GSH content ($p < 0.05$) without any alterations on TNF-α levels of the bladders with cystitis. Additionally, combination of different concentrations of CP and DMF exhibited a potent synergistic cytotoxic effect in SH-SY5Y cells. DMF improved CP-induced acute cystitis by partially suppressing oxidative stress and inflammation, while also enhancing the cytotoxic effects of CP.

1 | Introduction

Cyclophosphamide (CP) is an oxazaphosphorine agent widely used in the treatment of a variety of malignancies from lymphoma to breast cancer. In addition, due to its broad spectrum of action, it is clinically preferred as an immunosuppressive agent in the treatment of autoimmune diseases [1]. The widespread use of CP also leads to the frequent occurrence of its side effects. The major disturbing side effect of CP is urotoxicity, which can occur with short-and/or long-term use of CP [2]. Although cystitis symptoms are more common in women than men in general, urotoxic properties of CP under chemotherapy regimen regardless to gender [3, 4]. The mechanism of CP-induced cystitis is multifactorial and not well understood. However, numerous studies indicated that the corrosive metabolite of CP, acrolein, accumulates in urine and causes ulceration and urothelial damage in bladder tissue by activating apoptotic signaling. Moreover, acrolein initiates lipid

peroxidation, depletes antioxidant defense potential and triggers inflammation that leads to functional and histological changes in the bladder [2, 5, 6]. Urological complications of CP vary from dysuria, hematuria, decreased urinary frequency, abdominal pain and voiding dysfunction with pathophysiological changes in bladder, and these side effects may persist after discontinuation of therapy [7, 8]. Therefore, the most recommended treatment strategy for CP-induced cystitis is to prevent the occurrence of cystitis. Mesna (2-mercaptoethane sulfonate sodium) is the only FDA approved drug for the treatment of CP-induced hemorrhagic cystitis and prevent urinary damage by neutralizing urotoxic metabolite of CP, acrolein when administered before CP application [7, 9]. Chemotherapy-induced cystitis is an increasingly important clinical problem with challenging treatment, especially since more aggressive tendency should be considered in the treatment of resistant cancer types [10]. Some of the studies claimed that mesna treatment does not completely prevent chemotherapy-induced bladder damage [11–13]. Thus, there is an increasing tendency to discover new therapeutic options for the treatment of CP-induced cystitis.

Dimethyl fumarate (DMF) is an FDA-approved drug used to treat psoriasis and multiple sclerosis. DMF is a pro-drug that rapidly hydrolysed to its pharmacologically active metabolite, monomethyl fumarate [14]. Although the exact mechanism of action of these fumaric acid derivatives has not yet been fully elucidated, both fumarates have been reported to enhance the antioxidant defense mechanism in various cell types by acting through the nuclear factor erythroid 2-related factor 2 (Nrf2) [15, 16]. Additionally, DMF mitigates inflammation by inhibiting the expression and activity of the inflammatory transcription factor, NFκB in several pathological condition. As well as its direct effect on NFκB, Nrf2 activation via DMF also plays a major role in reducing the expression of inflammatory genes [14, 17]. Until now, accumulating evidence suggested that DMF could be a promising therapeutic candidate in various conditions where oxidative stress and inflammation play a vital role in disease progression [18–20]. DMF also exerts antitumor properties by inhibiting cancer cell viability, invasion, and migration across different cancer types. Besides its antitumor activity, DMF sensitizes chemotherapy-resistant cancer cells to standard chemotherapy regimens by modulating the expression of several genes involved in chemotherapy resistance [21, 22]. These findings pointed out that DMF has great potential as an adjuvant cytotoxic agent for the treatment of multiple resistant cancer cells.

In the present study, we aimed to explore the possible protective effect of DMF on CP-induced cystitis and determine whether this adjuvant effect would alter the cytotoxic properties of CP.

2 | Materials and Methods

2.1 | Animals

Healthy female Balb/C mice weighing 25–30 g (6–8 weeks old) were obtained and kept under standard experimental conditions (humidity-controlled rooms, 23°C ± 1°C; 12-h light-dark cycle). Mice were free to access water and fed a standard diet. The experimental protocol was approved by Institutional Animal Care and Ethics Committee (Approval number: 2024/29) and performed according to the Guide for the Care and Use of Laboratory Animals and ARRIVE guidelines have been followed for the experimental description.

2.2 | Experimental Groups and Treatment Protocols

Adult 108 mice were randomly divided into six groups (18 mice for each) as follow; 1-Control, 2- CP, 3- DMF-300, 4- DMF-100 + CP, 5- DMF-300 + CP, 6- Mesna+CP groups. While control and CP groups were treated with 0.5% w/v carboxymethylcellulose orally, the DMF groups were treated with low (100 mg/kg/day) or high (300 mg/kg/day) dose of DMF via gastric gavage for five consecutive days before cystitis induction (Figure 1).

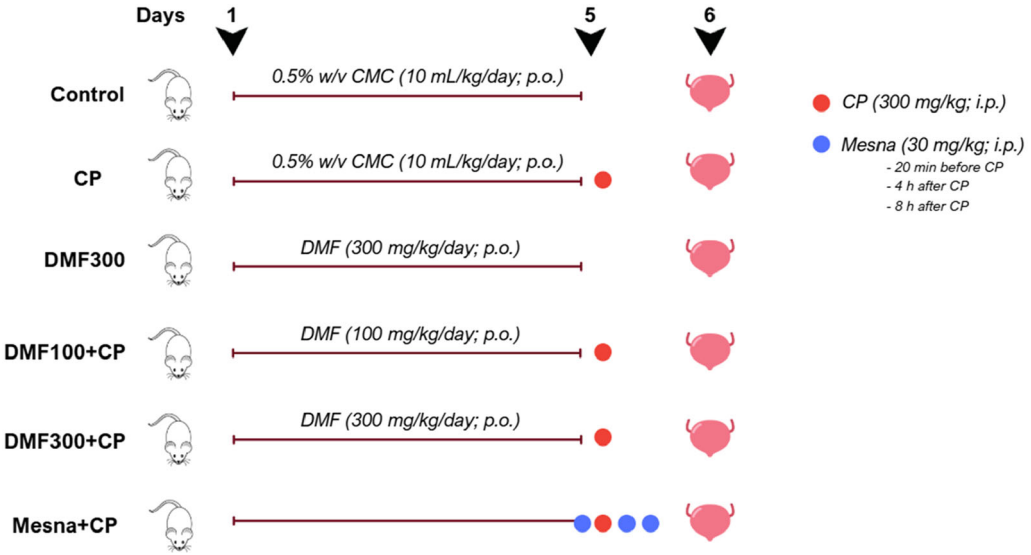

FIGURE 1 | The schematic representation of experimental design. CMC, carboxymethylcellulose; CP, cyclophosphamide; DMF, dimethyl fumarate.

On the fifth day, a single dose of CP (300 mg/kg) was injected intraperitoneally (i.p.) to establish an acute cystitis model. Mesna (30 mg/kg; i.p.) was administered 20 min before and 4, 8 h after CP injection. CP was dissolved in 0.9% sterile saline and DMF was suspended in 0.5% w/v carboxymethylcellulose. The dose of CP and DMF were selected based on previous in vivo studies with some modifications [14, 23, 24]. Twenty-four hours after CP injection, mice were euthanized by cervical dislocation under anesthesia and the whole urinary bladders were rapidly isolated, weighted and used for functional and Evans blue analysis or stored at −80°C until biochemical analysis.

2.3 | Detrusor Contractility

Urinary bladder from each mouse was gently removed and free from adherent tissues. After removal of the trigon and urinary sphincter, detrusor smooth muscle (DSM) strips (3–4 mm long and 2–3 mm wide) were prepared for the isolated organ bath studies as previously described [24, 25]. Strips were mounted longitudinally in 30 mL organ baths containing Krebs-Henseleit (mmol/L: NaCl 118, KCl 4.7, NaH_2PO_4 1.2, $MgSO_4$ 1.3, $CaCl_2$ 2.5, $NaHCO_3$ 25 ve glucose 11) solution and gassed with carbogen (95% O_2 and 5% CO_2) at 37°C.

Isometric tension was recorded via an isometric force transducer (MAY FDT10A, Turkiye) attached to an acquisition system (BIOPAC MP35, USA). The DSM strips were stabilized under 1 g resting tension with fresh Krebs-Henseleit solution washouts every 20 min for 60 min. Following the equilibration period, the tissue viability of DSM strips was verified by the KCl (80 mM)-induced contraction.

For functional analysis, carbachol (CCh, a muscarinic agonist) response curve was obtained by cumulative addition of CCh with one log increment (10^{-8}–10^{-4} M) for each strip. The contractile response to CCh was normalized to the wet tissue weight of the DSM strips. The maximal response to CCh (Emax) and the negative logarithm of the concentration to produce the half of maximal response (pEC_{50}) was calculated using nonlinear regression plots (GraphPad Prism version 5.0, GraphPad, San Diego, CA, USA).

2.4 | Assessment of Vesical Vascular Permeability

A distended bladder is an important indicator of increased vesical vascular permeability and edema. Thus, the bladder weight was measured to evaluate the impairment in bladder wall permeability.

To evaluate the impairment in vascular permeability of the bladder with cystitis, following 24 h CP injection, Evans blue (0.5% w/v in saline) was injected via tail vein into the mice. Thirty min later, the mice were killed, bladders were harvested, weighed, and then placed into 1 mL formamide solution. They were incubated at 56°C for 24 h. On the next day, samples were centrifuged at 14.000 x g for 45 min at 4°C. The formamide absorbance for each sample was measured with a UV-Vis spectrophotometer at 620 nm and quantified as microgram dye per milligram (μg/mg) tissue by comparing standard curve [26, 27].

2.5 | Biochemical Analysis

Each urinary bladder was sliced and homogenized in ice-cold phosphate buffered saline (pH = 7.4. Then samples were centrifuged at 10.000 x g for 30 min at 4°C to separate cell debris and supernatant. Total glutathione (GSH) content of urinary bladder homogenates was determined with a commercial GSH kit (Cayman Chemical-703,002, Ann Arbor, MI, USA), and the level of pro-inflammatory cytokine TNF-α was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen-88-7324, Waltham, MA, USA) according to the manufacturer's instructions. Total GSH content and TNF-α level were presented as μmol/mg protein and pg/mL, respectively [24].

2.6 | MTT Assay

Cell viability was assayed by MTT (3- (4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) test. Briefly, SH-SY5Y human neuroblastoma cell line (ATCC, CRL-2266) seeded on 48-well microplates and incubated for 24 h to observe a confluent layer as previously [28]. The following day, cells were exposed to different concentrations of doxorubicin HCL (Dox, 0, 1–10 μM), DMF (1–100 μM) dissolved in dimethyl sulfoxide (DMSO) at final concentration 0.5% (v/v). CP (10–1000 μM) was dissolved in complete DMEM. After 48 h of incubation, the prepared MTT was applied at a concentration of 0.5 mg/mL and incubated for an additional 2 h at 37°C. Following the incubation, 100 μL isopropanol was added to the wells and the absorption of MTT formazan was determined at 570 nm with a UV-spectrophotometric plate reader (Clariostar, Perkin Elmer). The absorbance of the cells exposed to CP, DMF and their combination for viability was defined as % cell viability by proportion to the viability of cells treated with negative solvent control. Synergistic cytotoxicity of combinations was calculated according to Bliss Independence Model (BIM) [29].

2.7 | Statistical Analysis

Data were expressed as the mean ± standard error (SEM). Statistical analyzes were performed using GraphPad Prism (Version 5.01; Graphpad Software, San Diego, CA, USA). The Kolmogorov–Smirnov test was used to determine whether data were normally distributed. The differences between groups were compared with ANOVA, followed by Bonferroni test. Student's *t*-test was used to compare two independent groups. $p < 0.05$ was considered statistically significant.

3 | Results

3.1 | Effect of DMF Treatment on DSM Contractility in CP-Induced Acute Cystitis

The CCh-induced contractions of DSM were significantly decreased in cystitis group compared to the control group

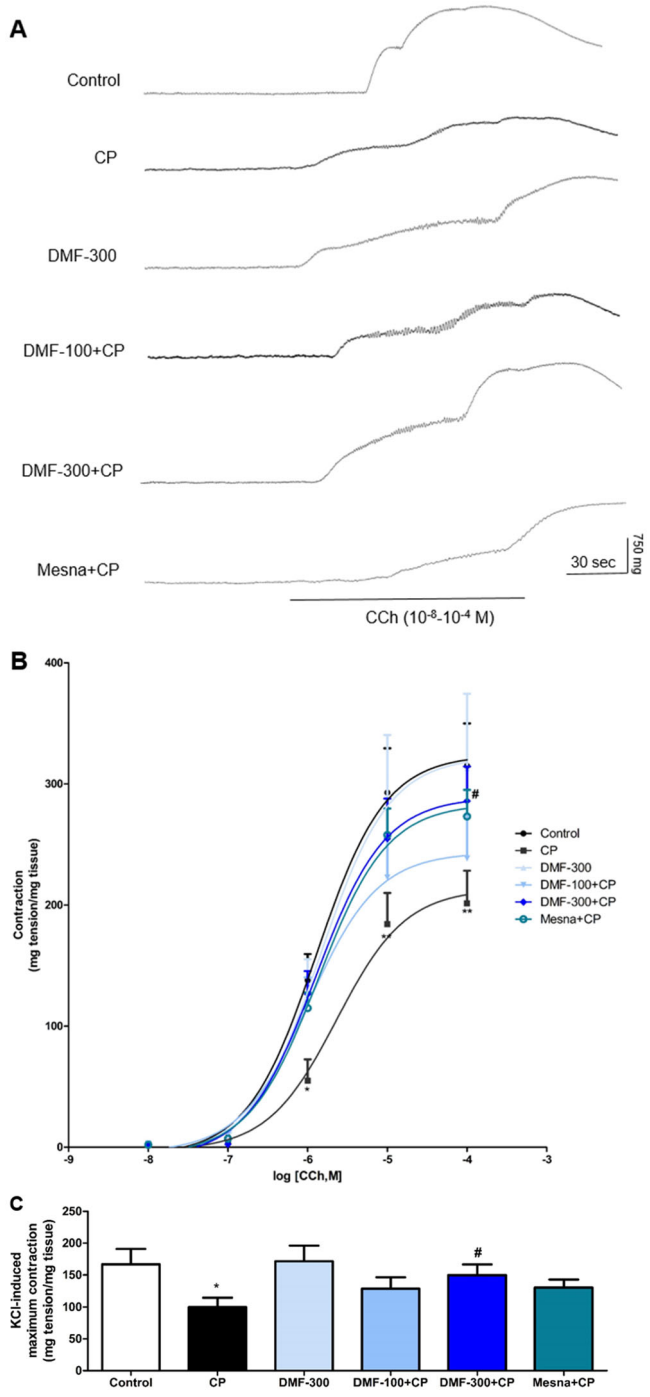

FIGURE 2 | DMF improved detrusor contractility in mice with CP-induced cystitis. (A) Representative traces of CCh-induced contractions of DSM strips for all groups. (B) Cumulative concentration-response curves for CCh. (C). Maximum contractile response induced by KCl. Data were expressed as mean ± SEM ($n = 5–7$). $^{**}p < 0.01$, $^{*}p < 0.05$ significantly different from the control, $^{\#}p < 0.05$ significantly different from the CP group. CCh, carbachol; CP, cyclophosphamide; DMF, dimethyl fumarate.

($p < 0.05$, $p < 0.01$, Figure 2, Table 1). DMF at 300 mg/kg without CP did not alter the contractile response of DSM strips. While DMF treatment at 100 mg/kg and mesna did not change the CCh-evoked contractile responses compared to control group, only DMF treatment at 300 mg/kg significantly prevented the decrease in CCh-evoked contractile response compared to mice with cystitis ($p < 0.05$, Figure 2A,B). pEC_{50} values were similar among the groups (Table 1).

TABLE 1 | Emax and pEC_{50} values of CCh-induced contractions of detrusor strips for all groups.

Groups	Emax (mg/mg tissue)	pEC_{50}
Control	314.9 ± 35.05	5.893 ± 0.16
CP	201.4 ± 26.94*	5.635 ± 0.20
DMF-300	313.0 ± 64.48	5.844 ± 0.25
DMF-100 + CP	238.5 ± 35.73	5.986 ± 0.20
DMF-300 + CP	285.8 ± 28.53	5.902 ± 0.15
Mesna+CP	273.2 ± 22.00	5.869 ± 0.11

Note: Data were expressed as mean ± SEM ($n = 5–7$).
*$p < 0.05$ significantly different from the control group.

KCl-induced contractile response of DSM strips also decreased in cystitis group ($p < 0.05$, Figure 2C,D). DMF treatment at 300 mg/kg markedly increased the KCl-induced maximum contraction compared to CP group ($p < 0.05$, Figure 2D).

3.2 | Effect of DMF on Bladder Weight and Evans Blue Extravasation in the Bladder With Cystitis

CP treatment significantly increased relative bladder weight compared to the control group, demonstrating the increased bladder edema caused by cystitis ($p < 0.001$, Figure 3). The relative bladder weight was similar between only DMF-treated group and the control group. While DMF treatment at 100 mg/kg did not prevent edema formation in the bladder, DMF treatment at 300 mg/kg and mesna treatment markedly decreased relative bladder weight compared to cystitis group ($p < 0.01$, $p < 0.05$ Figure 3).

Vesical vascular permeability was extremely damaged by CP treatment, proved by the increment in Evans blue content in the bladder ($p < 0.001$, Figure 3). The bladder Evans blue level of DMF-300 group was similar to the control, however, in cystitis group, DMF at 300 mg/kg significantly prevented the extravasation of Evans blue dye, indicating DMF preserved vascular permeability of the bladder that was damaged by CP application ($p < 0.01$, Figure 3). While as positive control, mesna also decreased CP-induced Evans blue increment in the bladder, low-dose DMF treatment (100 mg/kg) did not cause any changes ($p < 0.001$, $p > 0.05$, respectively, Figure 3).

3.3 | Effect of DMF on Total GSH and TNF-α Levels in Urinary Bladders of CP-Treated Mice

CP treatment markedly reduced endogenous total GSH content in the bladder ($p < 0.01$, Figure 4A), and only DMF treatment at 300 mg/kg and mesna treatment improved total GSH level compared to CP-treated mice, indicating that DMF augmented the antioxidant defense mechanism to overcome CP-induced bladder damage ($p < 0.05$, Figure 4A).

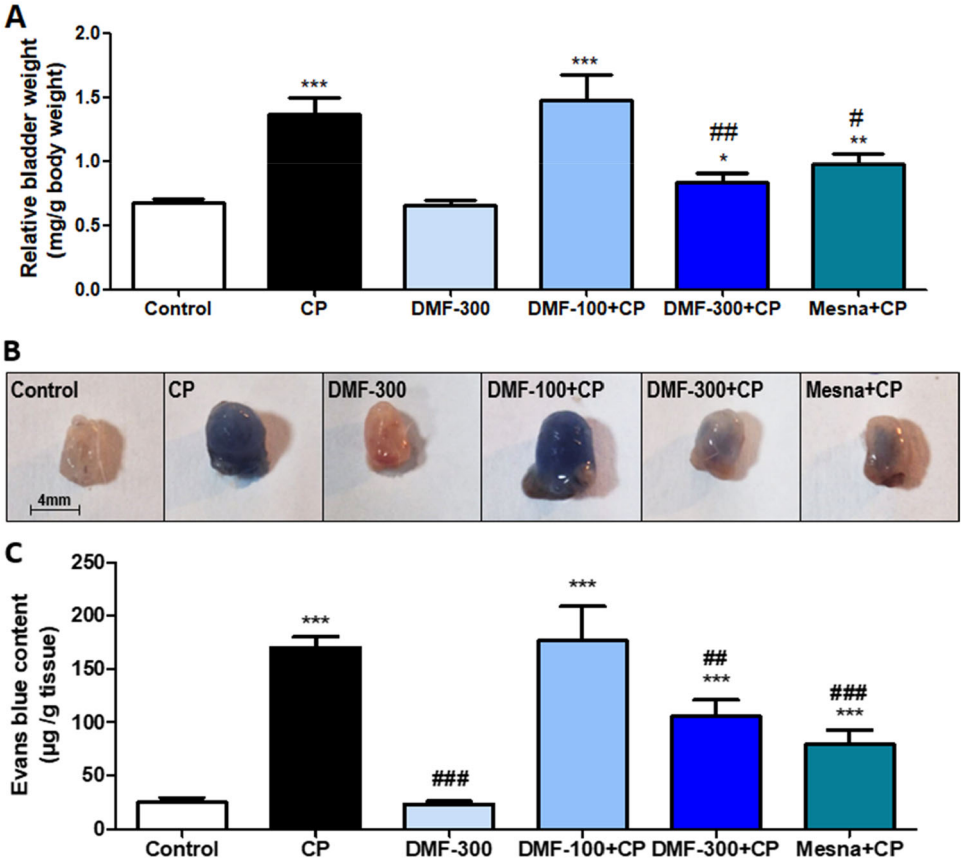

FIGURE 3 | DMF prevented bladder edema and Evans blue leakage in mice with CP-induced cystitis (A) Relative bladder weight of all groups (B) Typical images of CP-induced Evans blue dye extravasation into the bladder. (C) Evans blue dye content in the bladders from all groups. Data were expressed as mean ± SEM ($n = 6$–8). ***$p < 0.001$, significantly different from the control, ###$p < 0.001$, ##$p < 0.01$, significantly different from the CP group. CP, cyclophosphamide; DMF, dimethyl fumarate.

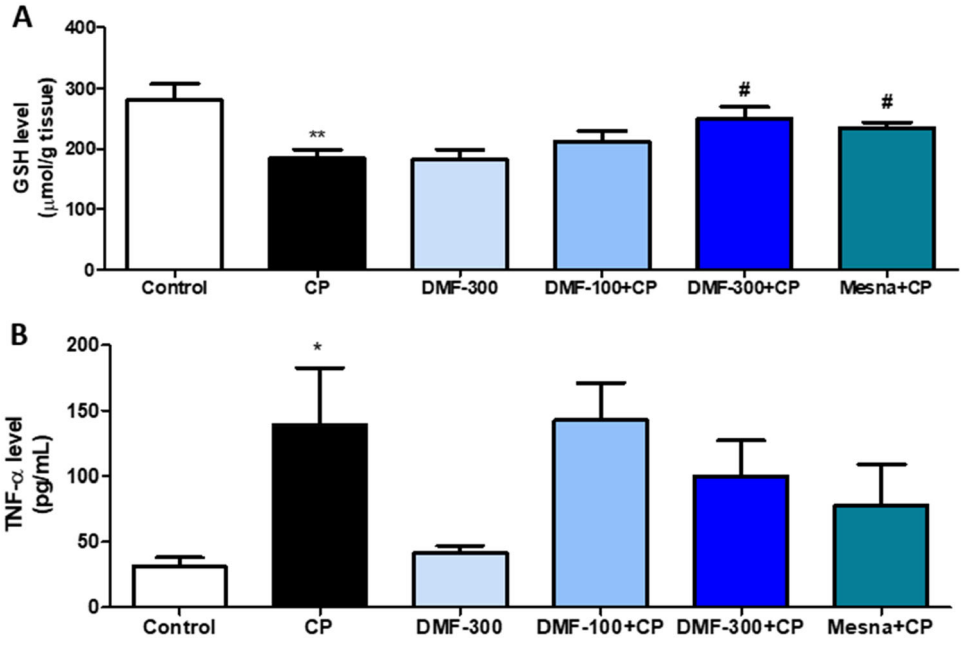

FIGURE 4 | Effect of DMF on GSH content and TNF-α levels in the bladders of mice with cystitis. (A) GSH level, (B) TNF-α level in the bladders from all groups. Data were expressed as mean ± SEM ($n = 4$–6). ***$p < 0.001$, significantly different from the control, ###$p < 0.001$, ##$p < 0.01$, significantly different from the CP group. CP, cyclophosphamide; DMF, dimethyl fumarate.

While CP-induced cystitis caused a significant increase in bladder TNF-α level, neither DMF nor mesna treatment was capable of preventing this inflammatory response ($p > 0.05$, Figure 4B).

3.4 | Synergistic Effect of DMF on the Cytotoxic Effect of CP in SH-SY5Y Cells

In SH-SY5Y cells, both CP and DMF showed concentration-dependent cytotoxicity for 48 h exposure (Figure 5A). Based on MTT assay, IC_{50} values of CP and DMF were 0.33 ± 0.3 μM and >100 μM, respectively. Moreover, combinations of different concentrations of CP (10–1000 μM) and DMF (1–100 μM) exhibited a potent synergistic effect in SH-SY5Y cells, particularly at combinations with above of 10 μM DMF (Figure 5B). Synergistic cytotoxicity of drugs according to MTT assay and BIM was shown in Table 2.

4 | Discussion

CP is an immunosuppressive and anticancer agent with high efficacy, used with a wide range of therapeutic conditions from autoimmune diseases to several solid tumors. For this reason, CP is commonly preferred in the clinic despite its toxic effects. High-dose CP treatment causes alopecia, myelotoxicity, liver and renal damage, gonadotoxicity and hemorrhagic cystitis [7, 30]. Hemorrhagic cystitis is a kind of noninfectious cystitis diagnosed nearly one in every four patients receiving high-dose CP treatment develops cystitis. In general, patients experience suprapubic pain, increased urinary frequency, urinary retention, nocturia and hematuria. Due to the lack of effective treatment options, CP-induced cystitis remains a significant clinical problem that negatively impacts the compliance of cancer patients [1, 6]. Thus, there is an urgent need to enhance the safety of CP in clinical practice by addressing the toxicities it causes, particularly its urotoxic side effects, and improving its cytotoxic effects against cancer cells.

CP-induced hemorrhagic cystitis originates from acrolein, an urotoxic metabolite accumulating in the bladder. Acrolein irritates the urothelium and mucosal membranes through the production of DNA adducts, leading to oxidative stress/inflammation and triggering apoptosis of urothelial cells [31, 32]. While the exact urotoxic etiology of acrolein is diverse, oxidative stress and inflammation are major contributors to the development of CP-induced cystitis [33]. CP-induced excessive production of reactive oxygen species disrupts the balance of oxidant-antioxidant system by decrease in GSH and other antioxidant enzyme levels [34]. In addition, our previous works implicated that high dose CP administration causes severe bladder damage and hemorrhage by disrupting antioxidant defense mechanisms and inducing NFκB-dependent inflammatory pathways, leading to an increased oxidative and inflammatory response [24, 25, 35]. In this regard, several studies have proposed that augmenting the antioxidant capacity to maintain the balance of the oxidant defense mechanism and decreasing the inflammatory response would be the main therapeutic targets for the treatment of chemotherapy-induced cystitis [36–38]. Therefore, we aimed to investigate the potential therapeutic effect of DMF on CP-induced acute cystitis due to its antioxidant and anti-inflammatory properties. Table 2.

DMF has become a subject of renewed research in the field of pharmacology due to altering the antioxidant gene's transcription factor Nrf2. Nrf2 activation is an essential cell-protective mechanism under aberrant oxidative stress conditions [20]. According to previous works, decreased Nrf2 expression and/or activity is associated with bladder dysfunction, and reducing oxidative damage by increasing Nrf2 activity could be a remarkable therapeutic target in severe bladder injury [39, 40]. In addition, Seidel et al. demonstrated that DMF significantly downregulated pro-inflammatory cytokines production via NFκB inhibition in airway smooth muscle cells [41]. Thus, we hypothesized that DMF, an Nrf2 activator, may ameliorate CP-induced acute cystitis and has synergistic activity with cytotoxic effect of CP against cancer cell due to its antioxidant and anti-inflammatory properties.

Consistent with previous studies conducted in our laboratory, we observed that a single dose of CP (300 mg/kg) significantly reduced CCh- and KCl-induced DSM contraction compared to the control group, indicating bladder dysfunction [24, 25, 35]. While positive control, mesna, treatment did not prevent the CP-induced bladder dysfunction, DMF at 300 mg/kg

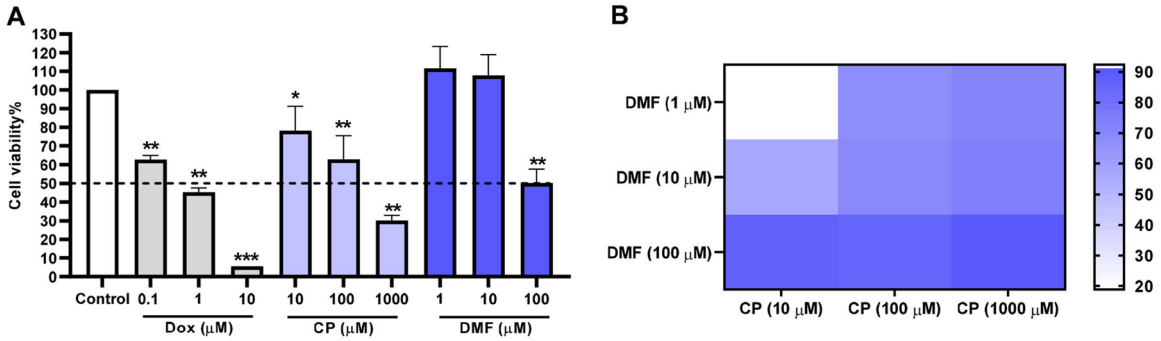

FIGURE 5 | Cytotoxicity of CP, DMF and their combinations in SH-SY5Y cells for 48 h-exposure. (A) Cytotoxicity of Dox, CP and DMF in SH-SY5Y cells according to the MTT assay. (B) Heatmap of inhibition rates of CP + DMF combinations at different concentrations in SH-SY5Y cells for 48 h. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ significantly different from the control. Control (DMSO 0.5%, v/v); CP, cyclophosphamide; DMF, dimethyl fumarate; Dox, doxorubicin HCl.

TABLE 2 | Synergistic cytotoxicity of drugs according to MTT assay and Bliss Independence Model.

CP (μM)	DMF (μM)	Viability inhibition rate % (MTT)	BIM inhibition rate %	Synergistic effect (Viability inhibition % > BIM inhibition%)
10	1	18.85 ± 0.3	21.64	No
	10	57.41 ± 6.3	21.64	Yes
	100	87.61 ± 8.5	57.48	Yes
100	1	68.76 ± 0.0	43.01	Yes
	10	70.55 ± 0.2	43.01	Yes
	100	86.51 ± 1.8	69.23	Yes
1000	1	72.56 ± 0.0	69.21	Yes
	10	75.16 ± 4.2	69.21	Yes
	100	92.33 ± 1.7	83.63	Yes

Abbreviations: CP, cyclophosphamide; DMF, dimethyl fumarate.

significantly improved DSM contractility in mice with cystitis. Amin et al. demonstrated that DMF treatment improved vascular reactivity to KCl and acetylcholine in diabetic rat aorta by suppressing the iNOS and NLRP3 inflammasome and enhancing eNOS expression [42]. CP-induced bladder damage was attributed to increased iNOS activity and inflammasome formation with a marked decrement in constitutive NOS activity. Moreover, the cholinergic innervation of the bladder also altered in CP-induced cystitis, resulting in bladder dysfunction [43, 44]. Nicoletti et al. reported that DMF treatment increased the central cholinergic activation in patients with multiple sclerosis. Although it is thought that DMF may mediate the activation of the cholinergic visceral pathway, findings to prove this hypothesis are quite limited [45]. All together, it can be predicted that DMF may differs the cholinergic pathway and/or NO signaling disrupted with CP application in the bladder. Further investigations are needed to clarify the exact mechanisms underlying the improved bladder contractility by DMF.

Evans blue extravasation into the bladder and the relative bladder weight are valid markers to evaluate the bladder inflammation and edema [46]. High-dose CP application markedly increased the relative bladder weight and Evans blue dye content, confirming that CP caused severe bladder damage. Only high-dose DMF (300 mg/kg) and mesna treatment significantly prevented Evans blue leakage into the bladder wall, as well as the increase in relative bladder weight. Several lines of evidence have indicated that high-dose CP treatment caused massive bladder edema and extravasation following bladder inflammation, while mesna treatment also decreased plasma extravasation in the bladder and prevented chemotherapy-induced bladder edema [47–49]. In addition, in line with our findings, previous studies indicated that DMF treatment decreased Evans blue vascular extravasation under ischemic and inflammatory conditions [50, 51].

Total GSH content used as a marker for antioxidant defense mechanisms in several conditions [52]. In addition, decreased total GSH content of the bladder represents a crucial mechanism underlying the CP-induced cystitis due to the diminishment of nonenzymatic antioxidants [53]. We found that total GSH content was significantly decreased in bladder tissue with CP-induced cystitis. Only high-dose DMF (300 mg/kg) and mesna treatment prevented The GSH level decrement. In agreement with our findings, recent works confirmed that CP caused severe bladder damage by enhancing reactive oxygen species and decreasing the expression and/or activity of enzymatic or nonenzymatic antioxidant defense systems [53, 54]. Nrf2 induces GSH synthesis by altering the expression of glutathione synthase. Therefore, Nrf2 activators have been shown to be one of the most favorable therapeutic agents to strengthen the cell against oxidative stress-induced damage and are called GSH-boosting agents [55]. So, the therapeutic effect of DMF in CP-induced bladder damage may be attributed to its Nrf2-activating potential.

Besides oxidative stress, inflammation is one of the major players in the development of CP-induced cystitis. Previous data have shown that various pro-inflammatory cytokines, such as TNF-α, are significantly increased within the bladder in CP-treated bladders, confirming the bladder inflammation induced by CP [38, 56]. Moreover, Gomes et al. showed that anti-TNF-α and anti-IL-1 treatment improved bladder damage histopathologically and emphasized especially that TNF-α and IL-1 are crucial mediators in understanding the etiology of CP-induced acute cystitis [57]. Therefore, we evaluated the TNF-α level of the bladder following CP and DMF treatment. We found that CP application dramatically increased bladder TNF-α levels. Although high-dose DMF and mesna treatment tended to decrease the CP-induced increase in TNF-α level, it was not significantly different. McGuire et al. found that DMF treatment caused a significant reduction in LPS-induced TNF-α secretion in bone marrow derived macrophages independently of the Nrf2-Keap1 pathway [58]. Another in vivo study demonstrated that DMF improved murine colitis by inhibiting IL-1β, TNF-α and IL-6 cytokine levels in colon tissues [59]. Although further studies are needed to explain our findings, when our results are evaluated together with the findings from the Evans blue assay, it can be predicted that DMF has the potential to suppress the inflammation regardless of TNF-α signaling in the bladder.

To claim that DMF could be used in CP-induced bladder dysfunction, it is also necessary to demonstrate that DMF does not reduce the cytotoxic effects of CP and ideally, its use as an

adjuvant agent for chemotherapy regimens should also be considered. To strength our results, we also aimed to demonstrate that besides uroprotective effect of DMF under CP treatment, it does not reduce the cytotoxic effect of CP. Growing evidence suggests that DMF directly reduces tumor cell growth and demonstrates significant cytotoxic activity in various cancer cell lines [60, 61]. In our work, DMF also reduced the viability of SH-SY5Y cells and its combination with CP showed potent synergistic effect against SH-SY5Y cells. Besides its uroprotective effect, DMF may also potentiate the cytotoxic properties of CP, making it a promising adjunct candidate for the patients under CP treatment in clinical settings.

5 | Conclusion

It is worth noting that, DMF treatment at 300 mg/kg ameliorated bladder contraction damaged by CP-induced acute cystitis and restored bladder damage by inhibiting oxidative stress and partially inflammatory response. The potential therapeutic mechanism of DMF in CP-induced cystitis appears to be multifactorial, thus further studies are needed to understand its potential as an effective adjuvant to cancer chemotherapy.

Author Contributions

Elif Nur Barut: conceptualization, methodology, data curation, formal analysis, supervision, writing – original draft, writing – review and editing. **Seçkin Engin:** methodology, data curation, formal analysis, writing – review and editing. **Elif Öz:** data curation, formal analysis, writing – review and editing. **Rengin Reis:** methodology, data curation, formal analysis, writing – review and editing.

Ethics Statement

The animal ethics application was approved by the Institutional Animal Care and Ethics Committee (Approval number: 2024/29) of Karadeniz Technical University.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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