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Effect of taxifolin on cyclophosphamide-induced oxidative and inflammatory bladder injury in rats

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Abstract: The role of oxidative stress and inflammation in the pathogenesis of cyclophosphamide-related side effects has been demonstrated in previous studies. This study aimed to investigate the effect of taxifolin, due to its antioxidant and anti-inflammatory properties, on cyclophosphamide-induced oxidative and inflammatory bladder injury in albino Wistar rats. The taxifolin+cyclophosphamide (TCYC) group was given 50 mg/kg of taxifolin orally by gavage. Normal saline was used as a solvent for the cyclophosphamide (CYC) group and the healthy control (HC) group. One hour after taxifolin administration, 75 mg/kg of cyclophosphamide was intraperitoneally injected in the TCYC and CYC groups. This procedure was repeated once a day for 30 days. At the end of this period, biochemical markers were studied in the excised bladder tissues and histopathological evaluations were conducted. In the histopathological evaluation of the CYC group, severe epithelial irregularity, dilatation, congestion, and polymorphonuclear leukocyte accumulation in the vascular structures were observed. Additionally, the malondialdehyde (MDA), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) levels, the total oxidant status (TOS), and the oxidative stress index (OSI) values were significantly higher, and the total glutathione (tGSH) levels and total antioxidant status (TAS) were significantly lower in the CYC group in comparison to the HC group (P<0.001). Taxifolin reduced the cyclophosphamide-induced increases in the MDA, TNF-α, IL-1β, and IL-6 levels and the TOS and OSI values; it decreased the tGSH and TAS levels and reduced histopathological damage (P<0.001). Taxifolin may be useful in the treatment of cyclophosphamide-induced bladder damage.
Key words: bladder, cyclophosphamide, inflammation, oxidative stress, taxifolin

Introduction

Cyclophosphamide is a nitrogen mustard derivative anticancer drug that is mainly used in the treatment of malignancies, such as Hodgkin lymphoma and Non-Hodgkin lymphoma, lymphocytic lymphoma, small lymphocytic lymphoma, Burkitt lymphoma, and multiple myeloma [1]. Breast cancer, diffuse neuroblastomas, retinoblastoma, and ovarian adenocarcinomas are also among the additional FDA-approved indications for its use [1]. As an effective immunosuppressive agent, cyclophosphamide is prescribed for the treatment of autoimmune diseases, such as multiple sclerosis, as well as before transplantation to prevent transplant rejection and graft-vs-host complications [2, 3]. However, nausea, bladder cancer, bone marrow suppression, increased opportunistic infections, and hematological and other organ toxicities have been observed during the use of cyclophosphamide [4]. Urotoxicity-like hemorrhagic cystitis has also been reported [1, 5]. One of cyclophosphamide's main metabolites, acrolein, is thought to be responsible for the drug's bladder toxicity, and Mesna is commonly

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used to counteract it [6, 7]. It is well known that, in some patients, Mesna does not properly protect the bladder from the harmful effects of cyclophosphamide. The increase in the level of malondialdehyde (MDA), a result of lipid peroxidation (LPO), and the decrease in the level of the endogenous antioxidant glutathione (GSH) have been demonstrated to cause the toxic effects of cyclophosphamide on the bladder. It has also been suggested that cyclophosphamide-associated cystitis is associated with the expression of proinflammatory cytokines, such as nuclear factor-κB (NF-κB), tumor necrosis factor-alpha (TNF-α), interleukin 1 beta (IL-1β), and IL-6 [1, 8]. According to evidence reported in the literature, antioxidant and anti-inflammatory medications may be beneficial in the treatment of cyclophosphamide-induced bladder injury.

Taxifolin (3,5,7,3′,4′-pentahydroxy-flavanone) whose effects against cyclophosphamide-induced oxidative and inflammatory bladder injury are investigated in our study, is an antioxidant flavonoid [9]. Taxifolin is found in the content of plants, such as milk thistle, onions, French maritime pine, and tamarind [10]. It has been proven that taxifolin has anti-inflammatory, antitumor, antimicrobial, and antioxidant properties [11]. It has been reported that the antioxidant effect of taxifolin is due to its inhibition of reactive oxygen species (ROS) production [12].

According to our extensive literature search, no previous studies have investigated the preventive effects of taxifolin against cyclophosphamide-induced oxidative and inflammatory bladder injury. Therefore, our study aimed to biochemically and histopathologically investigate the effect of taxifolin on cyclophosphamide-induced oxidative and inflammatory bladder injury in rats.

Materials and Methods

Animals

The animals we used in the study were supplied from Erzincan Binali Yıldırım University Medical Experiments Application and Research Center. A total of 18 male, albino Wistar rats weighing between 250 and 265 grams were used in the experiment. The animals were obtained from the Erzincan Binali Yıldırım University Medical Experimental Application and Research Center. The animals were housed and fed in a suitable laboratory environment at normal room temperature (22°C) under appropriate conditions (12 h of light and 12 h of darkness). Animal experiments were performed in accordance with the National Guidelines for the Use and Care of Laboratory Animals and were approved by the local animal ethics committee of Erzincan Binali Yıldırım University, Erzincan, Turkey (Ethics Committee No.: 2021/05, dated Dec 30, 2021).

Chemicals

The cyclophosphamide (a vial containing 1 g of powder solution for infusion) used in the experiments was supplied from EİP Eczacıbaşı (İstanbul, Turkey), thiopental sodium was supplied from I.E. Ulagay (İstanbul, Turkey) and taxifolin was obtained from Evalar (Moscow, Russia).

Experimental groups

The rats used in the experiment were divided into three groups: a cyclophosphamide (CYC) group, a taxifolin + cyclophosphamide (TCYC) group, and a healthy control (HC) group.

Experiment procedure

To implement this experiment, the TCYC group (n=6) was given 50 mg/kg of taxifolin orally by gavage. This dose of taxifolin has been used in animals before and has been found to be effective against oxidative liver damage [13]. Normal saline (0.9% NaCl) was used as the solvent for the CYC (n=6) and HC (n=6) groups. One hour after taxifolin and solvent administration, 75 mg/kg of cyclophosphamide was injected intraperitoneally in the animals in the TCYC and CYC groups. This procedure was repeated once a day for 30 days. At the end of this period, the animals in all three groups were killed with high-dose anesthesia (thiopental sodium 50 mg/kg) and the bladder tissues were removed. The MDA, total glutathione (tGSH), TNF-α, IL-1β, and IL-6 levels and the total oxidant status (TOS) and total antioxidant status (TAS) were determined in the removed bladder tissues. The bladder tissues were also examined histopathologically. The biochemical and histopathological examination results were compared between the three study groups and evaluated.

Tissue MDA and tGSH determination

After measuring the weights of the tissues, samples were homogenized immediately for use for MDA measurements. MDA measurements are based on the method used by Ohkawa *et al.*, which includes the spectrophotometric measurement of the absorbance of the pink-colored complex formed by thiobarbituric acid and MDA [14]. Briefly, 25 μl of tissue homogenate were mixed with 25 μl of 80 g/l sodium dodecyl sulfate and 1 ml of combination solution (20 g/l acetic acid + 1.06 g 2-thiobarbiturate + 180 ml distilled water). The mixture was incubated for 1 h at 95°C. The mixture was centrifuged for 10 min at 4,000 rpm after cooling. The

supernatant's absorbance was measured at 532 nm. The standard curve was obtained by using 1,1,3,3-tetramethoxypropane. tGSH measurement was made according to the method described [15].

Tissue TOS and TAS determination

The TOS and TAS results of tissue homogenates were determined using a novel automated measurement method and commercially available kits (Rel Assay Diagnostics, Gaziantep Turkey), both developed by Erel [16, 17]. The measurement of different oxidants and antioxidant molecules separately is not practical. Therefore, measuring the total oxidant or antioxidant capacities of a biological sample and relating them in a ratio, called index is preferred. One of the indexes used for this purpose is the "Oxidative stress index (OSI)". The OSI value calculated according to the "OSI (Arbitrary Units) = TOS (nmol H2O2 Eq/mg protein) / TAS (nmol Trolox Eq/mg protein) × 100" formula.

TNF-*α*, IL-1*β*, and IL-6 analysis

The weight of the tissue samples was measured; then the samples were rapidly frozen with liquid nitrogen and homogenized using a pestle and mortar. The samples were maintained at 2–8°C after melting. We added phosphate-buffered saline (pH 7.4), 1/10 (w/v). Then, the samples were vortexed for 10 s, centrifuged for 20 min at 10,000 × g and the supernatants were carefully collected. The levels of TNF-α (ng/l), IL-1β (pg/l), and IL-6 (ng/l) were measured using a commercial ELISA kit supplied by Eastbiopharm Co., Ltd. (Hangzhou, China).

Histopathological analyses

The tissue samples were fixed with 10% formaldehyde for 72 h. Following the fixation process, the tissue samples were washed under tap water in cassettes for 24 h. The samples were then treated with conventional grade alcohol (70%, 80%, 90%, and 100%) to remove the water within the tissues. The tissues were then passed through xylol and embedded in paraffin. Four-to-five micron sections were cut from the paraffin blocks and hematoxylin-eosin staining was administered. The sections were photographed and assessed using the DP2-SAL firmware program and a light microscope (Olympus Inc., Tokyo, Japan). From the serial sections taken, one central and five peripheral areas were selected for semi-quantitative scoring, and degeneration criteria were scored in the selected areas for each subject. Urinary bladder tissue damage was defined as the presence of epithelial necrosis, dilatation/congestion, polymorphonuclear leukocyte (PMNL) infiltration, and edema. Each sample was scored for each criterion as follows: 0: indicated no damage; 1: mild damage; 2: moderate damage; 3: severe damage. Histopathological assessment and scoring were performed by a pathologist who was blinded to the experimental groups.

Statistical analysis

IBM SPSS Statistics for Windows, Version 22.0, released 2013 (IBM Corp., Armonk, NY, USA) was used for the statistical analysis. The results are presented as mean ± SD. The significance of differences between the groups was determined using the one-way analysis of variance (ANOVA) test followed by Tukey's analysis. The statistical level of significance for all tests was considered to be 0.05.

Results

Biochemical findings

As can be seen from Table 1, the amount of MDA in the bladder tissue of the rats in the CYC group was significantly increased in comparison to the HC and TCYC groups, while the amount of tGSH decreased ($P<0.01$). There was no statistically significant difference between

Table 1. Biochemical findings of the HC, CYC, and CYC groups

Parameters	HC (Mean ± SD)	CYC (Mean ± SD)	TCYC (Mean ± SD)
MDA (*μ*mol/gr protein)	2.77 ± 0.17[a]	5.73 ± 0.35	2.97 ± 0.35[a]
tGSH (nmol/gr protein)	4.71 ± 0.19[a]	2.03 ± 0.66	4.47 ± 0.16[a]
TOS (nmol H_2O_2/mg protein)	3.23 ± 0.08[a]	6.04 ± 0.38	3.53 ± 0.31[a]
TAS (*μ*mol Trolox Equiv/mg protein)	3.88 ± 0.14[a,b]	1.69 ± 0.13	3.57 ± 0.30[a,b]
OSİ (Arbitrary Units)	0.08 ± 0.01a	0.36 ± 0.04	0.11 ± 0.02a
TNF-α (ng/l)	3.21 ± 0.08[a,b]	7.53 ± 0.10	3.79 ± 0.39[a,b]
IL-1β (pg/l)	2.54 ± 0.11[a]	5.55 ± 0.38	2.80 ± 0.14[a]
IL-6 (ng/l)	2.27 ± 0.06[a,b]	4.74 ± 0.11	2.43 ± 0.09[a,b]

HC: Healthy Control Group; CYC: Cyclophosphamide Group; TCYC: Taxifolin+Cyclophosphamide Group; MDA: Malondialdehyde; tGSH: Total glutathione; TOS: Total oxidant status; TAS: Total antioxidant status; OSI: Oxidative stress index; TNF-α: Tumor necrosis factor-α; IL-1β: Interleukin-1β; IL-6: Interleukin-6. "a" refers to the statistically significant difference of the groups with the CYC group ($P<0.001$). "b" refers to the significant statistical difference between HC and TCYC groups ($P<0.05$).

the HC and TCYC groups in terms of the MDA and tGSH levels (Table 1).

TOS, TAS, and oxidative stress index (OSI) analysis results

In the CYC group, cyclophosphamide induced an increase in the TOS value of the bladder tissue and it decreased the TAS value. The TOS level was lower in the HC and TCYC groups in comparison to the CYC group (P<0.001), and the TAS value was higher (P<0.001, Table 1). Furthermore, the difference in the TOS values between the HC and TCYC groups was determined to be insignificant, despite the fact that there was a significant difference in TAS (P=0.046, Table 1). The OSI value was found to be significantly higher in the CYC group than the HC and TCYC groups. However, the difference in the OSI value between the HC and TCYC groups was insignificant (P>0.05).

TNF-α, IL-1β, and IL-6 analysis results

TNF-α, IL-1β, and IL-6 levels in the bladder tissue of the animals in the CYC group increased significantly in comparison to the HC and TCYC groups (P<0.001, Table 1). Taxifolin significantly inhibited the increase in the TNF-α, IL-1β, and IL-6 levels induced by cyclophosphamide. The difference in the IL-1β levels was found to be insignificant, although there was a significant difference in the TNF-α and IL-6 levels between the TCYC and HC groups (P=0.002 and P=0.015 respectively, Table 1).

Histopathological findings

In the bladder tissues of the animals in the HC group, the stratified transitional bladder epithelium, connective tissue, muscle tissue underneath the epithelium, and the distribution and architecture of the blood vessels in the connective tissue and muscle tissue were all normal (Fig. 1). In the bladder tissues of the animals in the CYC group, in low magnification, epithelial dysregulation, grade-3 dilatation and congestion in the blood vessels in the underlying connective and muscle tissue, grade-3 polymorphonuclear leukocytes (PNL) accumulation in the blood vessels, and grade-2 PNL cell infiltration around the blood vessels were observed (Fig. 2). Furthermore, when the high magnification images of the CYC group were examined, the stratified transitional epithelium lost its continuity in some areas, and grade-3 necrotic cells appeared in the areas where it was continuous. In the connective tissue underneath the epithelium, grade-3 edema and fibril separations were identified (Fig. 3).

Conversely, in the samples from the TCYC group, the epithelium is continuous, the underlying connective tissue is free of edema, and the fibrils are in a normal arrangement. Mild-to-moderate (grade-1 and grade-2) dilatation and congestion in the blood vessels, as well as grade-1 polymorphonuclear cell infiltration (PMNL), were observed in the connective tissue (Fig. 4). Table 2 presents the statistical analysis of histopathological findings in the HC, CYC, and TCYC groups.

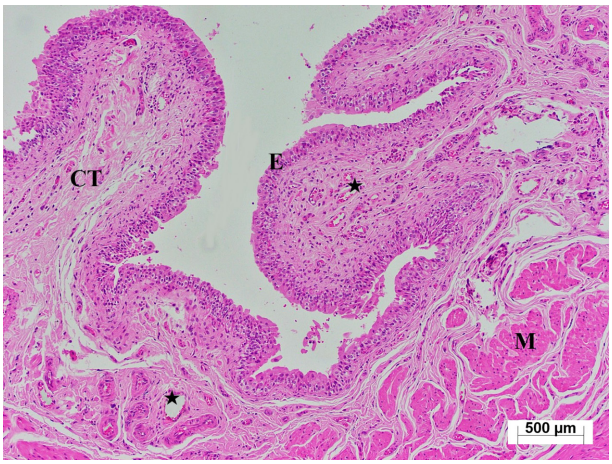

Fig. 1. Hematoxylin-eosin-stained bladder tissue belonging to the HC group; E: Stratified transitional epithelium, CT: Connective tissue, M: Muscle tissue, ★: Blood vessel, ×40.

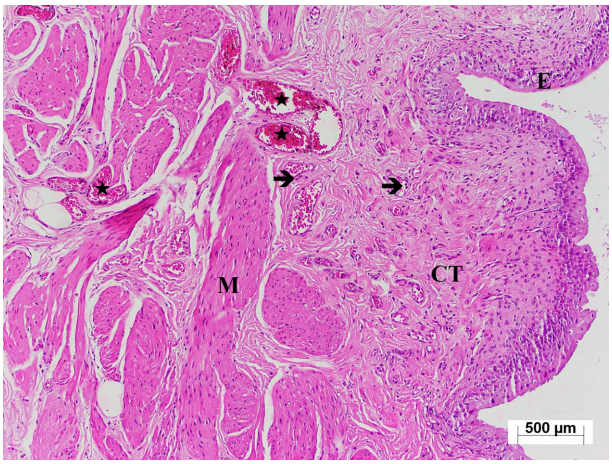

Fig. 2. Hematoxylin-eosin-stained bladder tissue belonging to the CYC group; E: Stratified transitional epithelium, CT: Connective tissue, M: Muscle tissue, →: Polymorphonuclear cell infiltration, ★: Severely dilated and congested blood vessels, ×40.

Discussion

In this study, taxifolin's effects on the oxidative and inflammatory bladder damage caused by cyclophosphamide in rats were studied biochemically and histopathologically. The balance between the oxidant and antioxidant systems in cyclophosphamide-induced oxidative

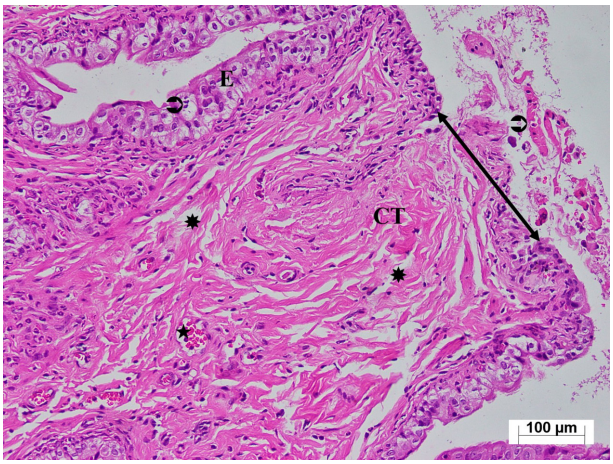

Fig. 3. Hematoxylin-eosin-stained bladder tissue belonging to the CYC group; E: Stratified transitional epithelium, ➲: Necrotic cells and debris, ↔: Epithelial detachments, CT: Connective tissue, ✷: Edematous areas of connective tissue, ★: severely dilated and congested blood vessels, ×200.

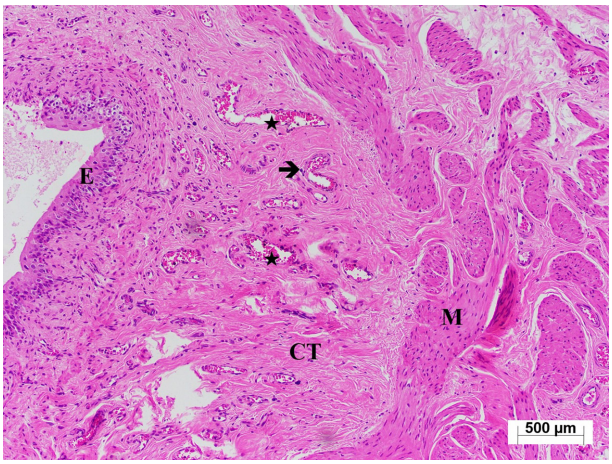

Fig. 4. Hematoxylin-eosin-stained bladder tissue of the TCYC group; E: Stratified transitional epithelium, CT: Connective tissue, M: Muscle tissue, →: Mild polymorphonuclear cell infiltration, ★: Mild to moderate dilated and congested blood vessels, ×40.

Table 2. Histopathological findings of the HC, CYC, and TCYC groups

Parameters	HC (Mean ± SD)	CYC (Mean ± SD)	TCYC (Mean ± SD)
Epithelial Necrosis	0.00 ± 0.00 [a,b]	2.64 ± 0.27	0.75 ± 0.27 [a,b]
Dilatation / Congestion	0.00 ± 0.00 [a,b]	2.69 ± 0.25	1.25 ± 0.48 [a,b]
PMNL Infiltration	0.00 ± 0.00 [a,b]	1.95 ± 0.31	0.50 ± 0.11 [a,b]
Edema	0.00 ± 0.00 [a,b]	1.69 ± 0.29	0.47 ± 0.27 [a,b]

HC: Healthy Control Group; CYC: Cyclophosphamide Group; TCYC: Taxifolin+Cyclophosphamide Group. "a" refers to the statistically significant difference of the groups with the CYC group (P<0.001). "b" refers to the significant statistical difference between HC and TCYC groups (P<0.05).

bladder injury has been studied previously [8]. The level of MDA was increased and the level of tGSH was decreased in the animals with cyclophosphamide-induced bladder tissue injury, according to Abraham *et al.* [18]. As previously stated, ROS initiates the LPO reaction in the cell membrane, which results in the creation of toxic products, such as MDA from lipids [19]. These findings suggest that cyclophosphamide may have increased the production of ROS in the bladder tissue. In a study supporting this, it was reported that cyclophosphamide increased the production of ROS in the bladder tissue [20]. The fact that the MDA levels in the bladder tissue were higher in the CYC group than the HC group indicates that our experimental results are consistent with the data reported in the literature [21].

It is known that cyclophosphamide, which causes oxidative stress in bladder tissue, reduces endogenous antioxidant levels [22]. The decrease in the amount of tGSH in the bladder tissue to which we applied cyclophosphamide shows that our experimental results are in agreement with previous studies. Tasdemir *et al.* reported that cyclophosphamide decreased the amount of tGSH and increases the oxidant parameters in the bladder tissue [23]. In the literature, an increase in the oxidant parameters and a decrease in the antioxidant parameters have been associated with ROS overproduction [24]. Superoxide, singlet oxygen, hydroxyl radical, and hydrogen peroxides are known types of ROS [25]. tGSH is an endogenous antioxidant with ROS scavenging ability. Disruption of the ROS/GSH balance results in the oxidation of biomacromolecules; this leads to cell cycle arrest and even cell death [26].

To evaluate total enzymatic and non-enzymatic oxidant and antioxidant parameters, TOS and TAS in the bladder tissue were analyzed and the OSI values were calculated. The TOS and TAS analysis results obtained from the CYC group and the HC group verify that the level of MDA was increased in the bladder tissue and the level of tGSH was decreased. As is known, TOS and TAS reflect the total effects of all antioxidants and oxidants in tissues [16, 17]. Our results and this information obtained from the literature reveal that the oxidant/antioxidant balance in the bladder tissue of the animals in the CYC group is maintained by the predominance of oxidants.

In our study, it was found that the levels of proinflam-

matory cytokines, such as TNF-α, IL-1β, and IL-6, significantly increased in the bladder tissue of rats in the CYC group in comparison to the HC group. In recent studies, it has been reported that the levels of TNF-α, IL-1β, and IL-6 in the bladder tissues of animals with hemorrhagic cystitis due to cyclophosphamide are increased in comparison to the healthy control group; it has also been confirmed histopathologically that these proinflammatory cytokines cause inflammation in the bladder tissue [27]. A recent study by Wróbel *et al.* revealed that TNF-α, IL-1β, and IL-6 are important factors in the pathogenesis of cyclophosphamide-associated cystitis [28]. There also appears to be a correlation between the increase in proinflammatory cytokines and the oxidant and antioxidant parameters in cyclophosphamide-induced bladder damage [27].

In our study, it was observed that significant histopathological damage developed in the bladder tissue of the CYC group, in which the oxidant and proinflammatory parameters increased and the antioxidants decreased. The animals treated with cyclophosphamide experienced severe dilation and congestion of the bladder blood vessels. It has been reported that cyclophosphamide causes congestion in animal bladder tissue [29]. However, no previous studies reported that cyclophosphamide caused vasodilation in animal bladder tissue, based on a histopathological examination. In a patient who was treated with cyclophosphamide, it was found that capillary dilatation developed in the bladder mucosa in the cystoscopy performed [30]. Many studies have supported our findings showing PMNL accumulation in the bladder tissue of the cyclophosphamide group. Abd-Allah *et al.* and Moraes *et al.* reported that cyclophosphamide causes intense PMNL infiltration in rat bladder tissue [29, 31]. As it is known, the accumulation of PMNL in tissues is an indicator of inflammatory reaction. In our study, edema, which is one of the main signs of inflammation, developed in the connective tissue of the bladder in the CYC group. Other studies have also reported this finding [22, 29, 32]. Apart from inflammatory markers, necrosis was determined in the epithelial cells of the bladder tissue of the CYC group. In recent studies, there was no information that cyclophosphamide causes necrosis in bladder epithelial cells. However, in a previous study, it was stated that cystitis caused by cyclophosphamide is characterized by detachment of the urothelium, edema, microvascular damage, and focal muscle necrosis [33].

In our study, no histopathological damage was observed except mild and moderate dilatation, congestion, and PMNL infiltration in the connective tissue of the TCYC group. Histopathological findings were associated with the TCYC group's MDA, tGSH, TNF-α, IL-1β, and IL-6 levels and its TOS and TAS values. It is known that the effect of taxifolin against oxidative stress is due to its inhibitory effect on ROS production [12]. It has also been proven that taxifolin has anti-inflammatory, antitumor, antimicrobial, and antioxidant properties [11]. No previous studies have investigated the antioxidant and anti-inflammatory effects of taxifolin on the bladder. It has been documented that taxifolin protects liver tissue from oxidative damage by inhibiting the increase in MDA and TOS and decreasing the tGSH levels and TAS values due to pazopanib, an anticancer drug [13]. Another study reported that taxifolin protected optic nerve tissue against oxidative and inflammatory damage of the platinum-derived anticancer drug [34]. It has been argued that the protective effect of taxifolin is due to the inhibition of MDA, TNF-α, and IL-1β overproduction [35]. It has also been shown experimentally that taxifolin does not have a significant effect on the normal oxidant-oxidant balance in healthy tissue [21].

Taxifolin has also been shown to have cytoprotective properties via activating nuclear factor erythroid-2 related factor 2 (Nrf2). In response to oxidative stress, Nrf2 stimulates the transcription of cytoprotective genes, which is regulated by the adaptor protein Kelch-like ECH-associating protein 1 (Keap-1). Physiologically, Nrf2 is present at a low level and is maintained through ubiquitination mediated by Keap-1. Through the Nrf2-dependent pathway, taxifolin increases the production of phase II antioxidant and detoxifying enzymes, which had a critical protective effect against DNA oxidative damage. Importantly, taxifolin enhances Heme oxygenase-1 expression by increasing Nrf2 cytoplasmic expression and nuclear translocation. Taxifolin may increase the expression of the Nrf2 gene and its nuclear translocation through epigenetic changes [36, 37].

Quercetin is also a flavonoid that exhibited anti-inflammatory, anti-apoptotic, anti-thrombotic, and antitumor properties. The administration of quercetin effectively reduced cyclophosphamide-induced toxicity in multiple organs, including the heart, bladder, gonads, and lungs, in experimental mice. Two prior investigations in the literature found quercetin to be protective against cyclophosphamide-induced hemorrhagic cystitis. These studies indicated that quercetin could help protect the bladder against cyclophosphamide urotoxicity by lowering MDA levels in the bladder and raising antioxidant decreased GSH levels in the bladder, indicating quercetin's antioxidant activity [38, 39].

As a result, cyclophosphamide caused oxidative and inflammatory damage in the bladder tissue of rats. Taxifolin attenuated cyclophosphamide-related oxidative and inflammatory bladder injury by inhibiting the increase

in the oxidants and proinflammatory cytokines and decreasing the antioxidants. The protective effect of taxifolin against cyclophosphamide-induced bladder damage has also been demonstrated histopathologically. Our experimental results indicate that taxifolin may be useful in the treatment of bladder damage due to the use of cyclophosphamide. However, histopathological studies at the molecular level are required to clarify the protective effect mechanism of taxifolin against cyclophosphamide-induced bladder toxicity. Furthermore, it is important to investigate the role of anti-inflammatory cytokines in the protective action mechanism of taxifolin.

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