Original Research

Phenethyl isothiocyanate protects against cyclophosphamide-induced nephrotoxicity via nuclear factor E2–related factor 2 pathway in rats

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Impact statement

Cancer treatment involves administering various toxic chemicals to eliminate cancer cells. The target organs of chemotherapeutics are the kidneys and the liver. Kidney is the most commonly affected organ in cyclophosphamide (CP)-related toxicity. Food supplements are an important part of cancer therapy. Phytochemicals have been extensively studied because they are easily available and have fewer side effects. Phenethyl isothiocyanate (PEITC) has been shown to have chemopreventive and antioxidant effects in various experimental models. The role of PEITC has not previously been studied in CP-induced kidney injury. We studied the beneficial effects of PEITC on CP-induced renal injury. We proved that pretreatment with PEITC prevented renal dysfunction and tissue injury through nuclear factor E2–related factor 2 (Nrf2), sirtuin 1 (SIRT1), and nuclear factor kappa B (NF-κB). Our study is the first to provide data regarding this subject and will provide insights of development of phytochemicals as an adjunct treatment in cancer patients.

Abstract

Phenethyl isothiocyanate (PEITC), a secondary metabolite in Cruciferous plants, exerts chemopreventive and antioxidant effects. However, its therapeutic potential in cyclophosphamide (CP)-induced nephrotoxicity is not clear. So, we focused to research on the effect of PEITC against renal toxicity caused by CP and its relationship to the Nrf2 signaling mechanism. Thirty female Wistar albino rats were allocated to three groups: control ($n = 10$), CP ($n = 10$), and PEITC-pretreated group (150 µmol/kg b.w. orally; $n = 10$). The antioxidant enzyme activities and levels of malondialdehyde (MDA), sirtuin 1 (SIRT1), glutathione-S-transferase (GST), nuclear factor E2–related factor 2 (Nrf2), nuclear factor kappa B (NF-κB), serum urea, and creatinine (Cr) were measured. In the CP group, serum urea and Cr, MDA, and NF-κB levels have risen, and the activities of antioxidant enzymes and SIRT1, Nrf2, and GST levels have reduced significantly ($P < 0.05$). PEITC diminished levels of Cr, urea, MDA, and NF-κB while it enhanced antioxidant enzyme activities and GST, Nrf2, and SIRT1 levels significantly ($P < 0.05$). Pretreatment with PEITC ameliorated kidney tissue injury. The renal protective effect of the PEITC was supported by the histological analysis of the kidney. PEITC prevented CP-induced nephrotoxicity by decreasing oxidative damage through Nrf2 and SIRT1 activation and NF-κB inhibition. Therefore, we have suggested that PEITC may be a useful agent for protection against CP-induced renal injury.

Keywords: Cyclophosphamide, nephrotoxicity, Nrf2, phenethyl isothiocyanate, sirtuin1

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Introduction

Chemotherapeutic agents have a toxic effect on cells that have active replication such as cancer cells. However, they adversely also affect proliferating somatic cells. For this reason, chemotherapeutics also have side effects on normal cells.[1] Cytotoxic drug–related nephrotoxicity is among the most frequently observed side effects of chemotherapy.[2] Although the division rate of kidney cells is not high, they are highly susceptible to toxic damage as they encounter a high blood flow, and they have the capacity to concentrate toxins.[3] Among drugs that most frequently cause nephrotoxicity are antimetabolites, alkylating drugs, and anthracyclines.[4] Cyclophosphamide (CP) is a highly potent, nitrogen mustard-type alkylating cytotoxic drug that is prevalently employed in clinics in the therapy of cancer and some other diseases.[5] CP is mostly metabolized in the liver to phosphoramide mustard and acrolein (ACR).[6] CP is associated with toxicities such as nephrotoxicity,[7] lung toxicity,[8] cardiotoxicity,[9] and hepatotoxicity.[10] It is stated that ACR causes toxicity by producing high amounts of reactive oxygen species (ROS) and interfering with the antioxidant defense mechanism.[11]

Nuclear factor E2–related factor 2 (Nrf2), a transcription factor, is found with its inhibitor known as Kelch-like

epichlorohydrin-associated protein 1 or INrf2 in physiological conditions. When oxidative stress increases, Nrf2 separates from INrf2, locates in the nucleus, and then activates the antioxidant response element.[12] Its activation leads to increased levels of antioxidant and phase II detoxifying enzymes that eliminate toxic reactive by-products formed in xenobiotic metabolism.[13,14]

Nrf2 activation in the presence of ROS causes an increased expression of sirtuin 1 (SIRT1) and activators.[15] SIRT1 is the most researched among the sirtuins, a member of NAD+-dependent protein deacetylases, which is demonstrated to regulate the inflammatory, stress response, apoptosis, and energy metabolism.[16–18] SIRT1 inhibits inflammation through deacetylation of the p65 subunit of the nuclear factor kappa B (NF-κB) and prevents the synthesis of other inflammatory factors.[19] Phenethyl isothiocyanate (PEITC) is a phytochemical of the Cruciferae family and has been declared to exert antioxidant and chemopreventive effects through Nrf2 activation.[20] We assessed the potential preventive effect of PEITC on CP-induced nephrotoxicity via the Nrf2/SIRT1 pathways.

Materials and methods

Experimental animals and design

We studied in compliance with the "Ethical Guidelines for Animal Use" after receiving approval from the Local Experimental Animals Ethics Committee of the İnönü University (decision date: 25 March 2019 and number: 2018/A-48). The rats were accommodated under a 12-h light/dark period (22°C–24°C) and were fed *ad libitum* and tap water.

The study included 30 female Wistar albino rats (6–8 weeks old, 150–250 g weight) and they were randomly separated into three groups (10 rats each): In the control group (C), rats received saline daily for seven days by gavage; in the second group (CP), rats have been injected single-dose CP (150 mg/kg) intraperitoneally; and the rats in the third group (CP + PEITC) received PEITC (150 µmol/kg; Sigma-Aldrich) daily for one week by gavage followed by CP injection.

Sample collection

The day following the CP injection, blood was collected under xylazine/ketamine anesthesia and it was centrifuged at 2000*g* for 10 min. Serum creatinine (Cr) and urea levels were determined. One of the kidneys was kept in 10% formaldehyde until histopathological examinations. The other kidney was homogenized in phosphate buffer (50 mM, pH 7.4), centrifuged at 15,000*g* and 4°C for 15 min, and used for biochemical analyses. Nuclear extracts were prepared using a nuclear extraction kit (Abcam). Hybrid Multi-Mode Microplate Reader (Biotek Synergy H1M, USA) was used for biochemical analyses.

Biochemical analyses

Protein quantification was performed by the Bradford method.[21] Kidney function was evaluated by the determination of serum levels of Cr and urea with Creatinine ELISA kit (SunRed Biotechnology Company) and Urea (blood urea nitrogen [BUN]) Colorimetric Assay Kit (Elabscience), respectively. Results were expressed as milligrams per deciliter for Cr and millimoles per liter for urea.

Assays of oxidative stress parameters

Malondialdehyde (MDA) (nmol/g wet tissue) measurement was performed with the procedure of Uchiyama and Mihara.[22] Superoxide dismutase (SOD) activity (U/mg protein) by the procedure of Sun *et al.*,[23] catalase (CAT) activity (U/mg protein) by the method of Aebi,[24] and glutathione peroxidase (GPx) activity (U/mg protein) by the method of Paglia and Valentine[25] were performed.

Assays of GST, NF-κB, Nrf2, and SIRT1 levels

Glutathione-S-transferase (GST) and SIRT1 levels were measured by rat ELISA kits (Bioassay Technology Laboratory) in the tissue supernatant. Nrf2 Transcription Factor assay kit (Abcam) and NF-κB p65 ELISA kit (Elabscience) were used for the measurement of nuclear Nrf2 (%DNA binding activity) and NF-κB (pg/mg protein) levels respectively.

Histopathological evaluation

The kidneys were fixed in formalin (10%) and then embedded in paraffin. The slices (5-µm-thick cut) were stained with hematoxylin and eosin. They were evaluated regarding vascular congestion, mononuclear cell infiltration, edema, hemorrhage, glomerular degeneration, sloughing into the lumen in tubule cells, and swelling in tubule cells. The microscopic alterations for each criterion were defined as none (0), mild (1), moderate (2), and severe (3). Leica DFC 280 light microscope and the Leica Q Win Image Analysis System were used for analysis.

Statistical analysis

The IBM SPSS Statistics 22.0 for Windows package program was used for statistical analysis. The results were expressed as mean and standard deviation (SD). For testing normality, the Shapiro–Wilk test was used. Analysis of variance (ANOVA) was used in the intergroup comparisons among parametric tests, and pairwise comparisons were made using the least significant difference (LSD) test. $P < 0.05$ was accepted as statistically significant.

The statistical analysis of the histological examinations was performed with the SPSS and MedCalc programs. The non-parametric Kruskal–Wallis ANOVA test followed by the Mann–Whitney *U*-test was performed. Results were presented as mean ± standard error (SE), and $P < 0.0001$ was accepted as statistically significant.

Results

PEITC inhibits CP-induced kidney dysfunction

CP increased serum levels of Cr and urea significantly. Pretreatment with PEITC significantly diminished kidney damage by reducing Cr and urea levels ($P < 0.05$) (Figure 1).

PEITC alleviates CP-induced oxidative injury

The MDA levels were markedly increased in the CP-induced group ($P < 0.05$). Pre-administration of PEITC caused a reduction in MDA content compared to animals intoxicated

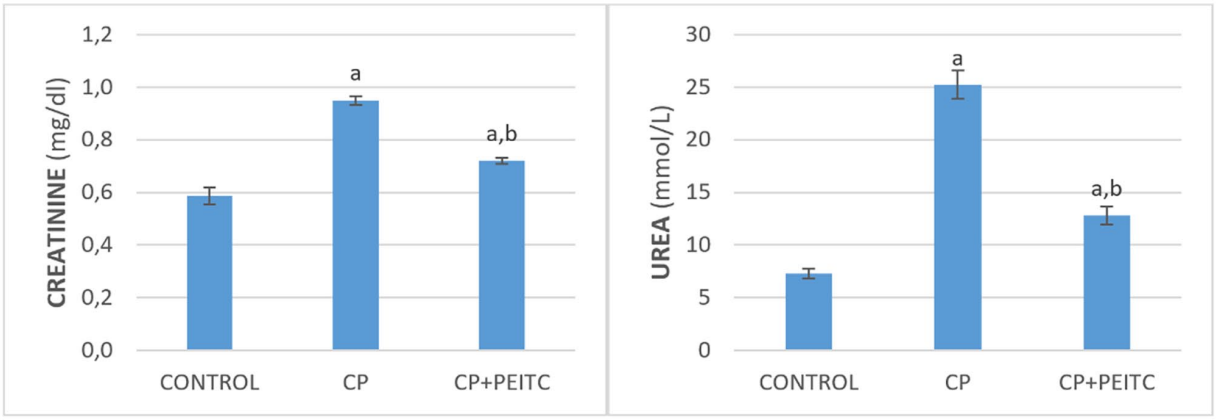

Figure 1. Effect of PEITC on kidney function markers against CP-induced kidney injury.
Data are displayed as mean ± SD.
[a]$P < 0.05$ compared to control; [b]$P < 0.05$ compared to CP.

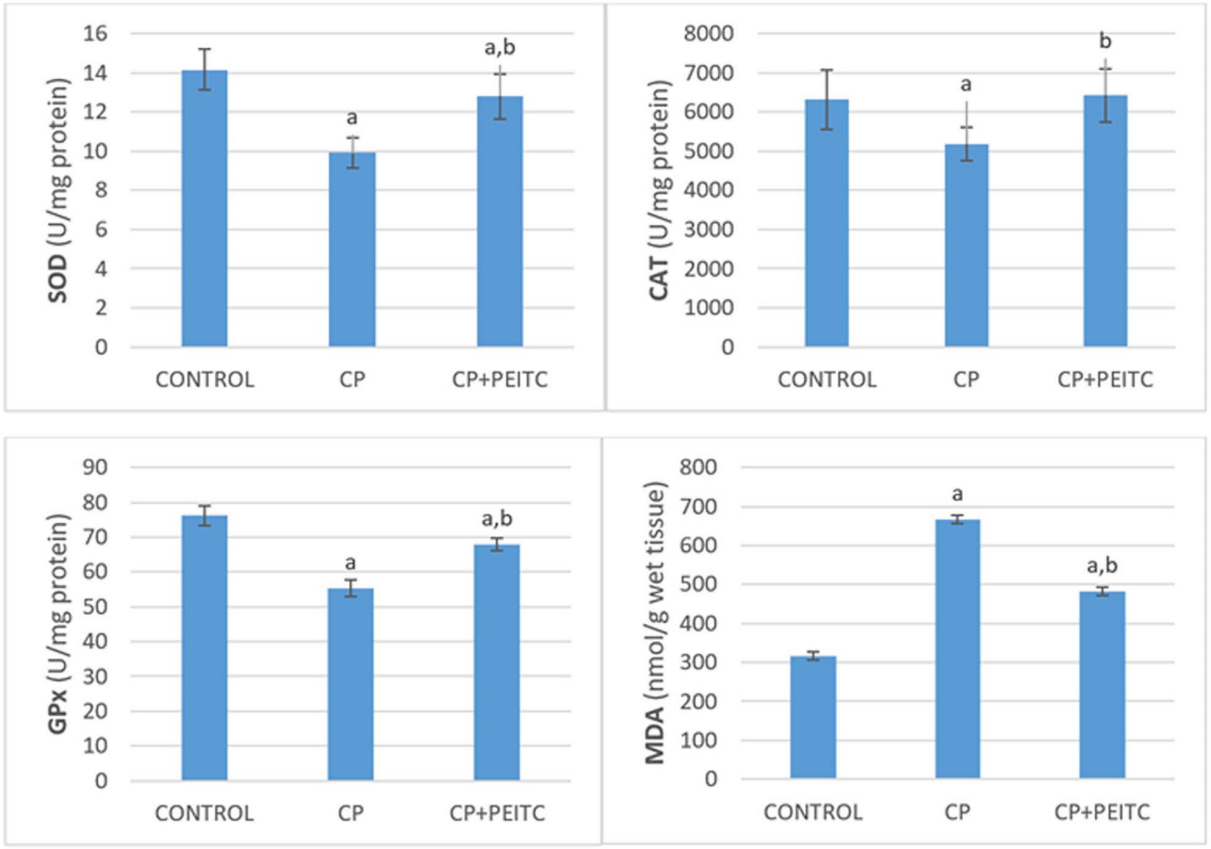

Figure 2. Effect of PEITC on oxidative stress parameters against CP-induced kidney injury.
Data are displayed as mean ± SD.
[a]$P < 0.05$ compared to control; [b]$P < 0.05$ compared to CP.

with CP significantly ($P < 0.05$) (Figure 2). CP exposure caused a reduction in SOD, GPx, and CAT activities. PEITC pretreatment before CP administration raised significantly the activities of SOD, GPx, and CAT ($P < 0.05$) (Figure 2).

PEITC pretreatment affects the levels of GST, SIRT1, NF-κB, and Nrf2

The GST level was reduced in the CP group significantly ($P < 0.05$). PEITC given led to a significantly increased level of GST according to CP-induced rats ($P < 0.05$) (Figure 3). The SIRT1 level was reduced in the CP-induced group significantly compared to the control group. PEITC led to a significantly increased level of SIRT1 compared to the CP-induced rats ($P < 0.05$) (Figure 3). Nrf2 activity was lessened after CP administration significantly ($P < 0.05$). PEITC treatment showed a significant elevation in Nrf2 activity by comparison with the CP-induced group ($P < 0.05$) (Figure 3). The level of NF-κB was significantly elevated in the CP group compared to control.

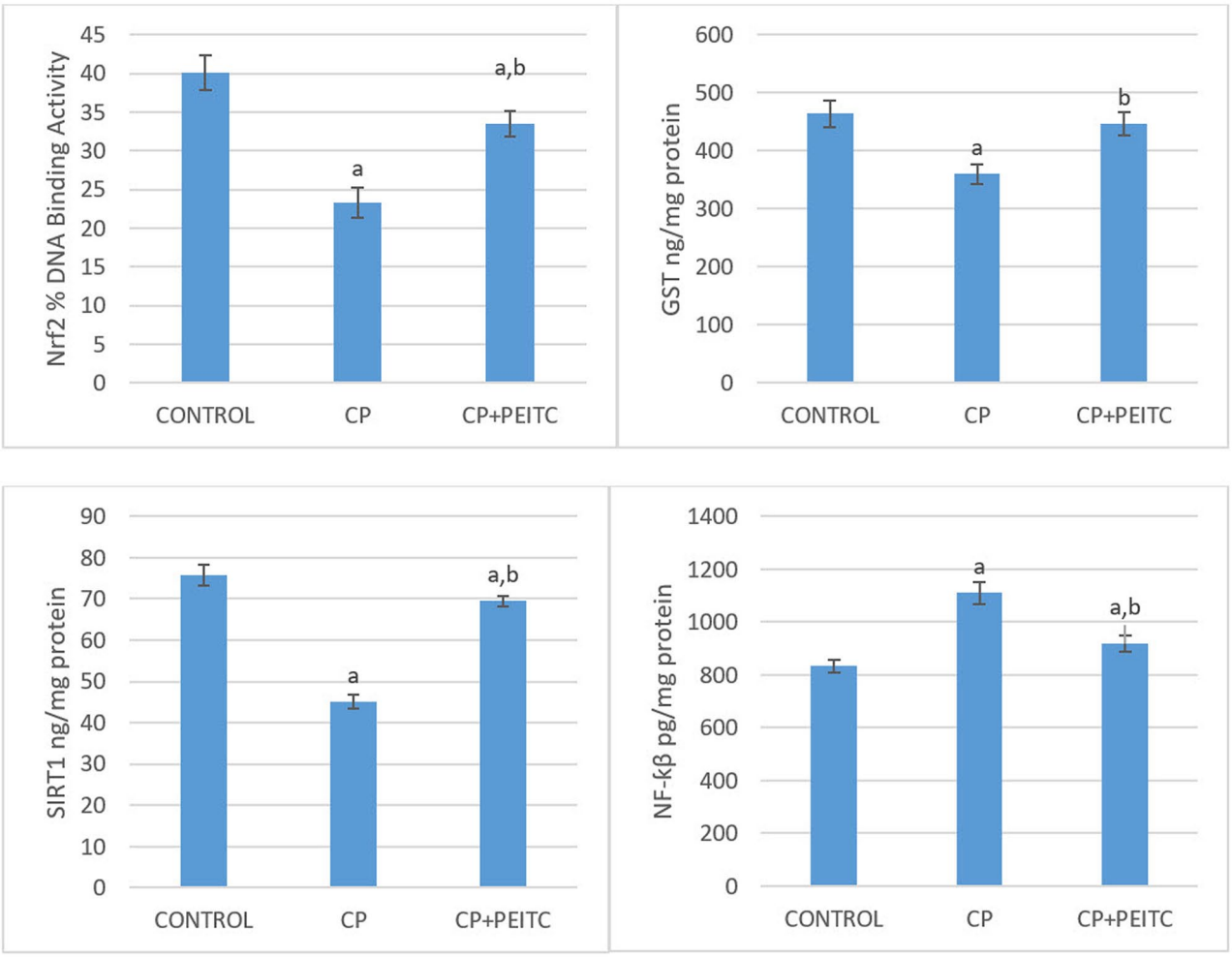

Figure 3. Effects of PEITC on the activity of Nrf2 and levels of GST, SIRT1, and NF-κB against CP-induced kidney injury.
Data are displayed as mean ± SD.
[a]$P < 0.05$ compared to control; [b]$P < 0.05$ compared to CP.

Pretreatment of rats with PEITC significantly reduced NF-κB levels ($P < 0.05$) (Figure 3).

PEITC reduces histopathological changes evoked by CP-induced injury

The pathologic analyses of the samples showed that the control group had a normal histological structure of renal tubule and glomerular cells (Figure 4(A) and (B)). In the CP group, vascular congestion (Figure 5(A) and (B)), mononuclear cell infiltration (Figure 5(A), (C), and (D)), edema (Figure 5(B)), hemorrhage (Figure 5(D) and (F)), glomerular degeneration (Figure 5(A) to (C), (E)), tubule cell shedding (Figure 5(E)), and swelling of tubule cells (Figure 5(F)) were observed. In the CP + PEITC group, histopathological damage in the kidney tissue was significantly reduced. A slight (mild) mononuclear cell infiltration (Figure 6(A) and (C)) and hemorrhage (Figure 6(C)) were observed. ($P < 0.001$). The renal damage score of the groups was control group (0.57 ± 0.11^{a}), CP group (1.83 ± 0.14^{b}), and CP + PEITC group (1.07 ± 0.09^{c}), respectively. (Mean ± SEM $n = 7$; the lowercase letters (a, b, c) indicate the differences between the groups, $P < 0.0001$).

Discussion

Drug-induced nephrotoxicity is a common side effect of cancer chemotherapeutics. Perfusion abnormalities, excess production of ROS, and inflammation are the major responsible mechanisms that contribute to drug-induced renal damage.[26] CP causes the generation of ROS, membrane lipid peroxidation, protein denaturation, and DNA damage which results in cellular damage and necrosis.[27,28] The reason for toxicity is its toxic metabolites such as ACR that increase the amount of free radicals and disrupt the antioxidant system.[29] Studies are investigating the efficacy of protecting effects of some natural products against nephrotoxicity induced by CP.[30–33] PEITC is a secondary metabolite in cruciferous vegetables, which is produced through hydrolysis of gluconasturtiin by myrosinase.[34] It is reported that PEITC has antioxidant and anti-inflammatory effects.[35] But there has been no report on the use of PEITC on CP-induced renal toxicity yet. This is the first study evaluating the efficacy and mechanism of action of PEITC in preventing CP-induced nephrotoxicity.

Mechanisms that lead to drug-induced nephrotoxicity include urine sediment anomalies, electrolyte imbalance,

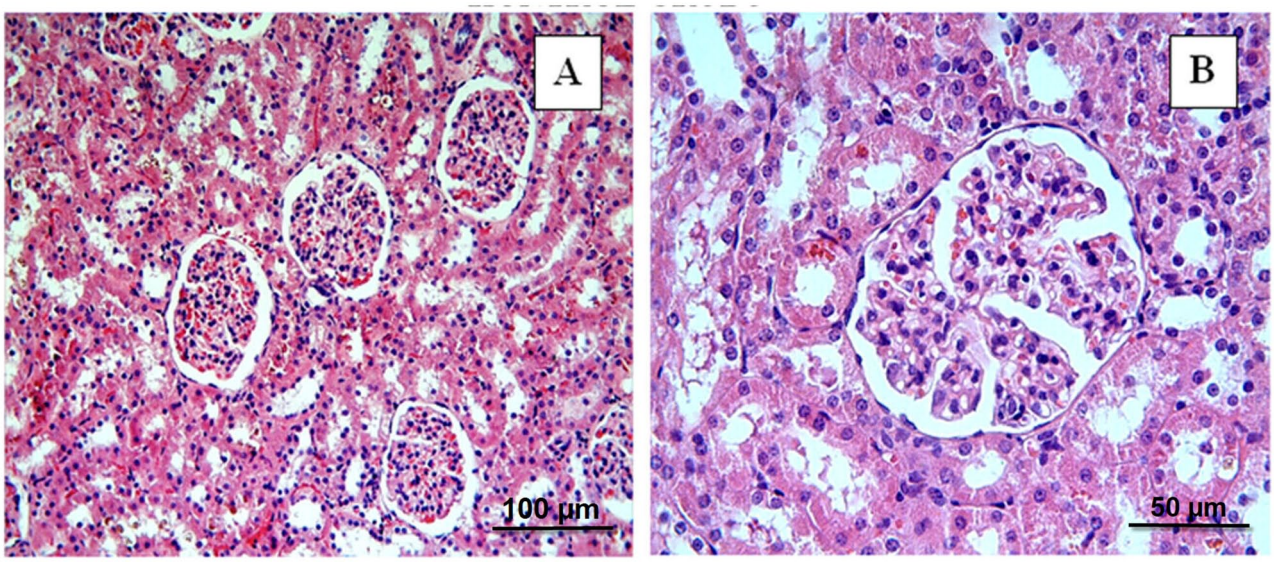

Figure 4. (A, B) Normal histological appearance of kidney tissue of control group (kidney tubules and glomeruli). (A) H&E, ×20 (bar = 100 µm) and (B) H&E, ×40 (bar = 50 µm).

and a decrease in the rate of glomerular filtration.[36] In our research, Cr and urea levels were elevated in the rats that were administered CP according to the control group. This difference among the groups may be attributed to renal glomerular and tubular damage. The treatment with PEITC reduced serum Cr and urea levels. Our histopathological results supported that PEITC decreased kidney injury. Therefore, we have clearly shown that PEITC is effective in preventing CP-induced nephrotoxicity in rats.

In this investigation, CP administration led to an elevation of MDA and a reduction in GPx, SOD, and CAT, together with a significant decrease in the GST level. Our results showed that PEITC protected the adverse effects of CP on renal functions. The reversal of kidney injury seems to be associated with decreased MDA and elevated antioxidant enzyme levels in rats which are pretreated with PEITC. Nrf2 is a principal transcriptional factor, and it actuates by oxidative stimuli caused to cell damage and inflammation.[37] Thus, the activation of Nrf2 signaling is accepted as a significant way of increasing antioxidant defense and protecting the cell. In our study, we showed that PEITC pretreatment activated the Nrf2 pathway and also increased antioxidant enzyme activities. Furthermore, PEITC diminished the MDA levels remarkably checked with the CP group. Thus, our data are the proof of concept that Nrf2 activation causes activation of antioxidant and cytoprotective pathways.

It was shown that numerous natural products with antioxidant attributes have favorable effects in repairing nephrotoxicity through Nrf2 activation in various kidney injury models.[38] Fan *et al.*[39] reported that iso-orientin showed a protective effect through the SIRT1/SIRT6/Nrf2 pathway against Cisplatin-induced nephrotoxicity. In addition, it was presented that pyrroloquinoline quinone reduced oxidative kidney damage in CP-induced nephrotoxicity through Nrf2 activation.[40] Some studies have demonstrated that PEITC has a cytoprotective effect via Nrf2 activation.[41,42] The results of our study pointed out that CP injection led to reducing the Nrf2 activity and it was significantly increased by PEITC administration.

Our study represented that SIRT1 and Nrf2 activation and NF-κB inhibition contribute to the preventive efficacy of PEITC against CP-induced renal toxicity. SIRT1 regulates various signal pathways and assists in the defense against oxidative stress.[43] SIRT1 may also prevent inflammation by downregulating NF-κB activity.[44] A study by Jung *et al.*[45] also showed that NF-κB p65 acetylation is regulated through SIRT1, and they argued that SIRT1 activation may be a probable target in repairing cisplatin-induced kidney damage. In addition, Tian *et al.*[46] informed that toxicity caused by CP was prevented via regulating the Nrf2/HO-1 and TLR4/NF-κB signaling. We showed that in the CP-treated group, Nrf2 activation and SIRT1 levels decreased and NF-κB levels increased, whereas PEITC pretreatment caused the activation of Nrf2 and led to an elevation in the SIRT1 levels and inhibition of NF-κB.

Our data demonstrated that PEITC was able to reduce the CP-induced kidney damage by activation of Nrf2 signaling. Based on data, we can state that activation of Nrf2 and SIRT1 pathways, and NF-κB inhibition may be the target mechanisms for CP-induced nephrotoxicity and are prevented by PEITC pretreatment. The protective effects of PEITC on other organ systems such as lungs, liver, and bone marrow, which are possible sites of adverse effects of chemotherapeutics, should also be investigated. PEITC can be a useful therapeutic tool for cancer patients to prevent the adverse effects of chemotherapeutics. Furthermore, concomitant or post-exposure treatment with PEITC should also be investigated with further experimental studies.

Conclusions

According to our findings, PEITC administration may have useful and renoprotective effects on CP-induced kidney damage. PEITC affected presumably the Nrf2/SIRT1

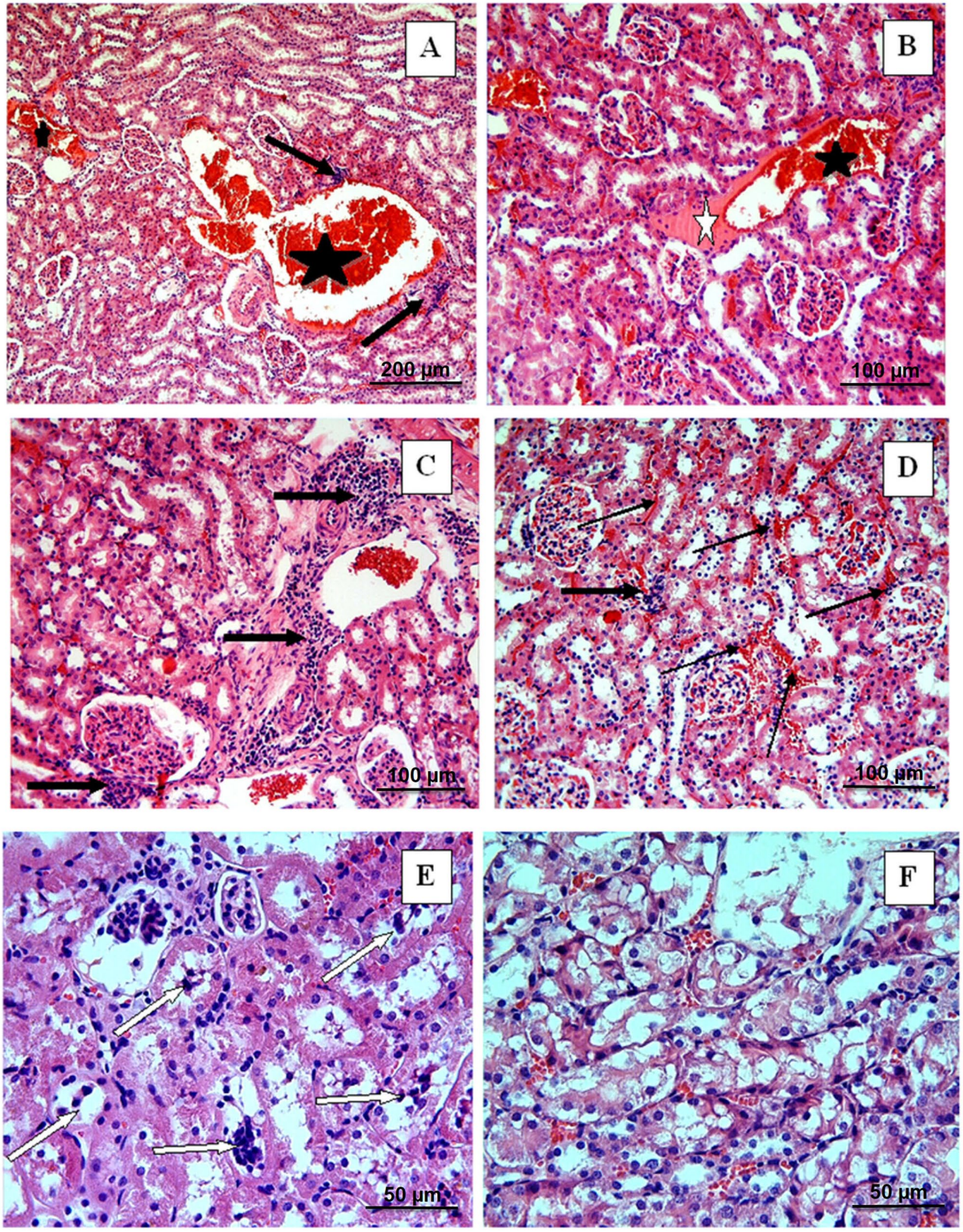

Figure 5. (A to F) CP group. Vascular congestion (black star) (A, B), mononuclear cell infiltration (black arrows) (A, C, D), edema (white star) (B), hemorrhage (black thin arrows) (D, F), glomerular degeneration (A, B, C, E), spillage of tubule cells into its lumen (white arrows) (E), and swelling of tubule cells (F) were observed. (A) H&E, ×10 (bar = 200 µm), (B to D) H&E, ×20 (bar = 100 µm), and (E, F) H&E, ×40 (bar = 50 µm).

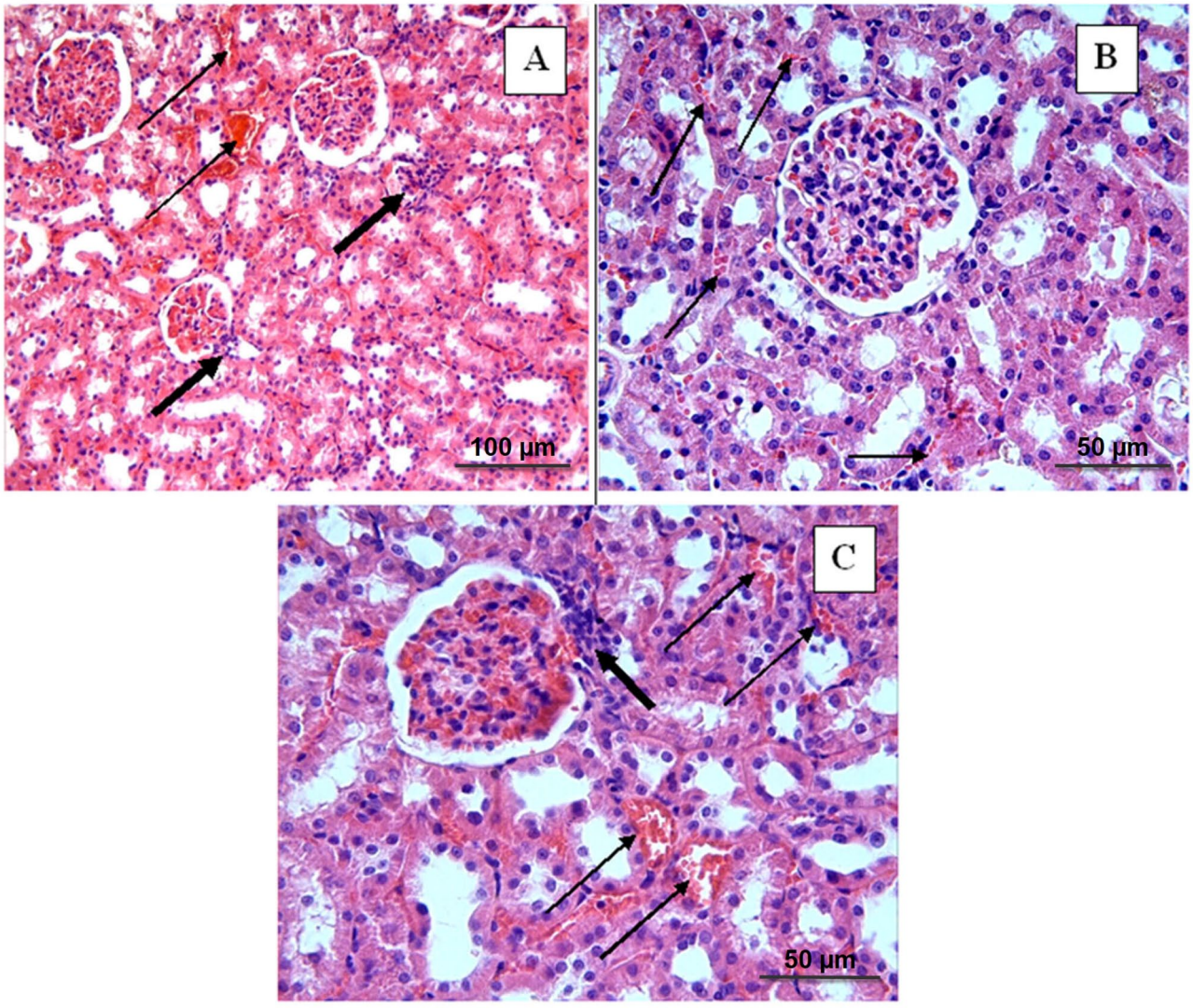

Figure 6. (A to C) Decrease in histopathological damages in the CP + PEITC group. We observed little mononuclear cell infiltration (black arrows) (A, C) and hemorrhage (black thin arrows) (C). (A) H&E, ×20 (bar = 100 µm) and (B, C) H&E, ×40 (bar = 50 µm).

pathway and interfered with some events including oxidative stress and inflammation processes in renal tissues.

AUTHORS' CONTRIBUTIONS

ABU directed the project, planned the experiments, and prepared the manuscript. ABU, BS and BA carried out the experiments. AT performed histopathological analysis. All authors read and accepted the final article.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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