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Renoprotective Effect of Formononetin against Cyclophosphamide-Induced Oxidative Stress and Inflammation in Rat Kidney

Saleem H. Aladaileh[1,2*], Farhan K. Al-Swailmi[1], Mohammad H. Abukhalil[2,3] and Mohammed H. Shalayel[1]

[1]Department of Pharmacy Practice, College of Pharmacy, University of Hafr Al-Batin, Hafr Al-Batin 31991, Saudi Arabia.
[2]Department of Medical Analysis, Princess Aisha Bint Al-Hussein Faculty of Nursing and Health Sciences, Al-Hussein Bin Talal University, Ma'an 71111, Jordan.
[3]Department of Biology, Faculty of Science, Al-Hussein Bin Talal University, Ma'an 71111, Jordan.

Authors' contributions

All authors contributed equally to this work. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Cyclophosphamide (CP) is a broad-spectrum chemotherapy agent available to treat various malignancies; however, its nephrotoxicity limits its clinical use. Formononetin (FOR) is a bioactive isoflavone with encouraging biological activities. The current study explored and elucidated the possible protective/therapeutic effects of formononetin against CP-induced nephrotoxicity.
Methodology: Rats received FOR (40 mg/kg/day) for 15 days followed by a single injection of CP on day 16. CP-induced nephrotoxicity is characterized by an increase in urea and creatinine levels in serum. Kidney homogenate was used to assess MDA, NO and antioxidants.
Results: CP-administered rats showed increased renal malondialdehyde and nitric oxide along with declined glutathione and antioxidant enzymes. In addition, CP increased pro-inflammatory cytokines and pro-apoptotic proteins levels and decreased anti-apoptotic protein Bcl2 levels in the kidney. FOR prevented CP-induced kidney injury, enhanced antioxidants and suppressed oxidative stress, pro-inflammatory mediators and apoptosis.

**Corresponding author: E-mail: shmoud@uhb.edu.sa;*

Conclusion: These findings suggest that FOR prevents CP nephrotoxicity by attenuating the oxidative damage and inflammation. Therefore, our data suggest that FOR may represent a novel protective strategy against CP-induced nephrotoxicity, which deserves pursuit in further studies.

Keywords: Formononetin; cyclophosphamide; ROS; nephrotoxicity; inflammation; antioxidant enzymes; isoflavone.

1. INTRODUCTION

Cyclophosphamide (CP), an alkylating agent, is one of the most potent chemotherapeutic agents used to treat various human malignant tumors [1,2]. Unfortunately, some limitations have been made on its clinical application due to its nephrotoxicity, which restricts the usage of high doses to maximize the therapeutic efficacy [3-5]. Exposure to high doses of CP causes injuries to normal tissue, and peroxidative damage to kidney and other vital organs by elevating the reactive oxygen species (ROS) [6]. Consequently, ROS result in lipid peroxidation, protein carbonylation and oxidative DNA damage [7,8], and that might lead to activation of multiple signaling nephrotoxicity pathways including pro-inflammatory cytokines [5,9], thereby amplifying the inflammatory cascade and eventually culminating in cell death. It is well-known that oxidative stress and inflammation play key roles in the activation of apoptotic signaling pathways in the kidney, which include mitochondrial-dependent caspase pathway [6]. Since oxidative stress plays a key role in the development of CP nephrotoxicity, mitigating oxidative stress and inflammation may protect against drugs-induced nephrotoxicity.

Many studies have proven the renoprotective effects of several antioxidants in animal models of CP-induced nephrotoxicity [6,10,11]. Formononetin (FOR, 7-Hydroxy-4'-methoxyisoflavone) is a bioactive isoflavone that can be extracted from the red clover (*Trifolium pratense*) and *Astragalus membranaceus* [12]. It has been proven to possess various medicinal benefits such as anti-inflammatory, antioxidant, anti-apoptotic and anti-tumor activities [12-16]. FOR was shown to protect against cisplatin-induced nephrotoxicity by enhancing proliferation of surviving renal tubular cells and suppressing apoptosis [17]. FOR has also been found to protect against rhabdomyolysis-induced renal apoptosis in vivo and in vitro by up-regulating Nrf2 [18]. In the same context, Nrf2 activation has been contributed to the protective effect of FOR against methotrexate-induced nephrotoxicity in rats [19]. A recently available study showed that FOR maintained kidney function by attenuating of ROS excessive generation and restoration of antioxidants in a rat model of type 2 diabetic nephropathy [20]. Furthermore, FOR demonstrated some anti-tumoral actions in a myeloma model by regulating and controlling many oncogenic cascades and gene products [21].

Although the antioxidant, anti-inflammatory and cytoprotective effects of FOR against a number of pathological conditions have been well described, its ability to protect against CP-induced oxidative stress and inflammation in the kidney has not been investigated. Our study therefore aimed to investigate the possible protective effect of FOR against CP nephrotoxicity, particularly focusing on oxidative stress and inflammation. Our findings may have significant implications for the prevention of the CP-induced nephrotoxicity.

2. MATERIALS AND METHODS

2.1 Animals and Drug Treatment

Male albino Wistar rats, weighing 200–220 g, were used in this study. Rats were acclimatized for one week before conducting the experiment. Rats were maintained in standard cages with a controlled environment at constant temperature (25 ± 2^{o}C) with a 12 h light/dark cycle. Animals had free access to food and water at all times. In order to fulfill the requirements of conducting animal experiments, the protocols involving the use of animals were followed in accordance with the guidelines of the National Institutes of Health (NIH publication No. 85-23, revised 2011).

According to the study protocol, four groups of rats (n = 6 rats in each group) were employed to study the protective effects of FOR against CP-induced kidney injury as given below:

Group I (Control): Control rats.
Group II (FOR): Rats received FOR (40 mg/kg) orally for 15 days [19].
Group III (CP): Rats received CP (150 mg/kg) on day 16 [22].

Group IV (CP + FOR): Rats received 40 mg/kg b. wt. FOR orally for 15 days and 150 mg/kg b. wt. CP on day 16.

CP was obtained from Baxter Oncology GmbH (Halle, Germany). CP was dissolved in physiological saline to reach a final concentration of 150mg/ml, and then administered intraperi toneally (i.p.). FOR was obtained from Sigma (MO, USA) and dissolved in 0.5% carboxymethyl cellulose (CMC) and the concentration was adjusted to be 40mg/kg, and administered via oral gavage. Groups I and III were given 0.5% CMC orally for two weeks, and groups I and II were given i.p. injections of physiological saline on day 16.

On the 19th day, the animals were anaesthetized using ketamine/xylazine, and then blood was drained and collected by cardiac puncture. The blood was left to coagulate, and sera were separated and used biochemical analyses. Kidney tissues were removed and cleaned from blood with cold phosphate buffered saline (PBS). The samples were then homogenized (10% w/v) in cold PBS and centrifuged. The resulted homogenate was used for biochemical parameters assessment.

2.2 Biochemical Assays

Serum levels of urea and creatinine were measured following the reagent kits instructions (Spinreact, Spain). Kidney homogenate was used to assess MDA, NO and antioxidants. The malondialdehyde (MDA) levels, were assessed by coupling of MDA with thiobarbituric acid as previously described by Ohkawa et al. [23]. The levels of NO were immediately measured as reported by Green et al. [24]. Reduced glutathione (GSH) content and superoxide dismutase (SOD) and catalase (CAT) activities in the kidney homogenate samples were measured following the previously described methods [25-28]. Tumor necrosis factor alpha (TNF-α), interleukin-1beta (IL-1β) and IL-6 levels in the kidney were determined using ELISA kits (R&D Systems, USA). The B-cell lymphoma 2 (Bcl2) and Bcl-2-associated X protein (Bax) levels in the kidney were estimated using ELISA kits provided by Cloud-Clone Crop (Houston, USA). Caspase-9 levels in the kidney were determined using an ELISA kits procured from Cusabio (Wuhan, China). All assays were performed following the manufacturer's instructions.

2.3 Statistical Analysis

GraphPad Prism 7 software (San Diego, CA, USA) was used for statistical analysis of the results. The results are reported as mean ± standard error of the mean (S.E.M). The statistical comparisons among groups were determined by one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. The results of P value < 0.05 were considered significant.

3. RESULTS

3.1 FOR Prevents CP-Induced Renal Dysfunction in Rats

To investigate the effect of FOR on CP-induced renal dysfunction, urea and creatinine levels in serum were investigated. As shown in Fig. 1A and B, CP caused a significant elevation in urea and creatinine levels in serum as compared to those obtain from the control group. Pre-treatment with FOR attenuated the CP-induced kidney dysfunction, with no effect on the kidney of normal animals (Fig. 1A and B).

3.2 FOR Pre-Treatment Attenuates Increased Oxidative Stress and Boosts Antioxidants Defenses in Kidney of CP-Intoxicated Rats

Because of CP-induced nephrotoxicity is known to be associated with increased oxidative stress, we evaluated the effect of FOR on MDA and NO. CP induced a significant increase in renal MDA (Fig. 2A) and NO (Fig. 2B) levels. In addition, the CP-induced increased oxidative stress was largely attenuated by FOR as compared to control group. CP administration also resulted in a significant decrease in renal GSH contents (Fig. 3A) and catalase (Fig. 3B) and SOD (Fig. 3C) activities, which were markedly attenuated by FOR treatment. FOR alone had no effects on the above-mentioned variables.

3.3 FOR Suppresses the CP-Induced Inflammation in Rats

CP significantly increased TNF-α, IL-1β and IL-6 levels in kidney compared to those from control group (Fig. 4A-C). FOR markedly attenuated the CP-induced elevation of TNF-α, IL-1β and IL-6 levels in the kidney. In FOR (40 mg/kg) treated rats, all the assayed inflammatory markers were not significantly affected.

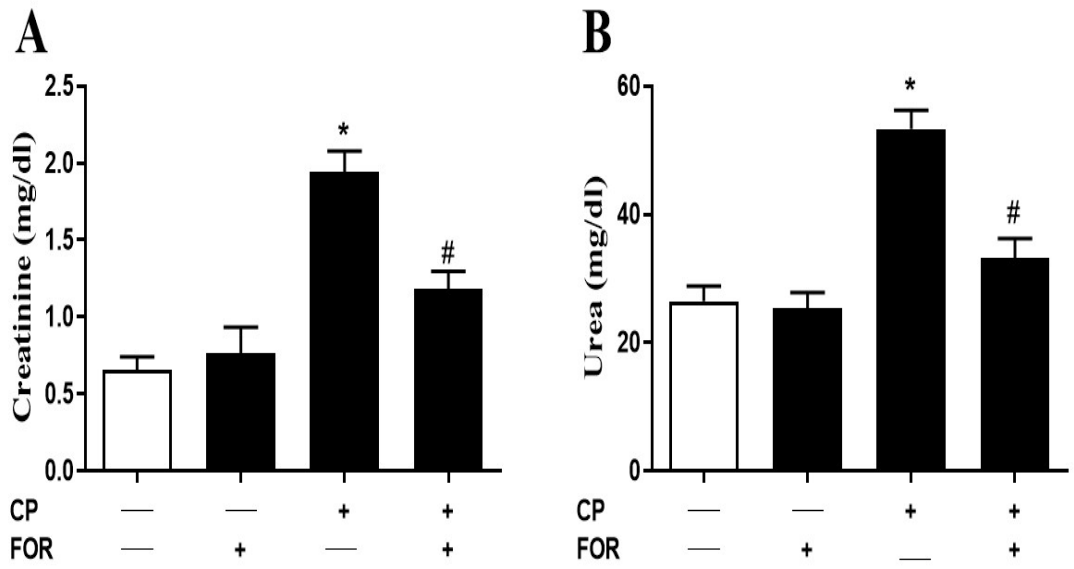

Fig. 1. FOR prevents the CP-induced kidney dysfunction

*CP administration induced a significant increase in (A) creatinine and (B) urea levels in serum. On contrary, the FOR treatment of CP- intoxicated rats ameliorated (A) creatinine and (B) urea levels in serum. Data are shown as Mean ± S.E.M, n=6. * P < 0.05 versus control group. # P < 0.05 versus CP group. FOR: Formononetin; CP: Cyclophosphamide*

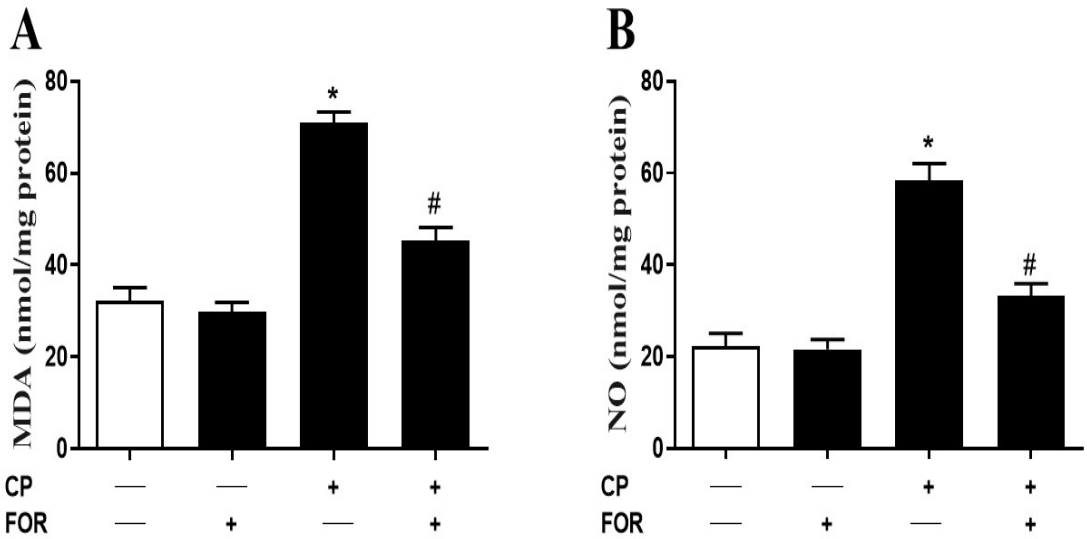

Fig. 2. FOR ameliorates the CP-induced renal oxidative stress

*CP administration induced a significant increase in (A) MDA and (B) NO levels in the kidney. Interestingly, treatment of CP-intoxicated rats ameliorated levels of (A) MDA and (B) NO in the kidney. Data are shown as Mean ± S.E.M, n=6. * P < 0.05 versus control group. # P < 0.05 versus CP group. FOR: Formononetin; CP: Cyclophosphamide; MDA: Malondialdehyde; NO: Nitric oxide*

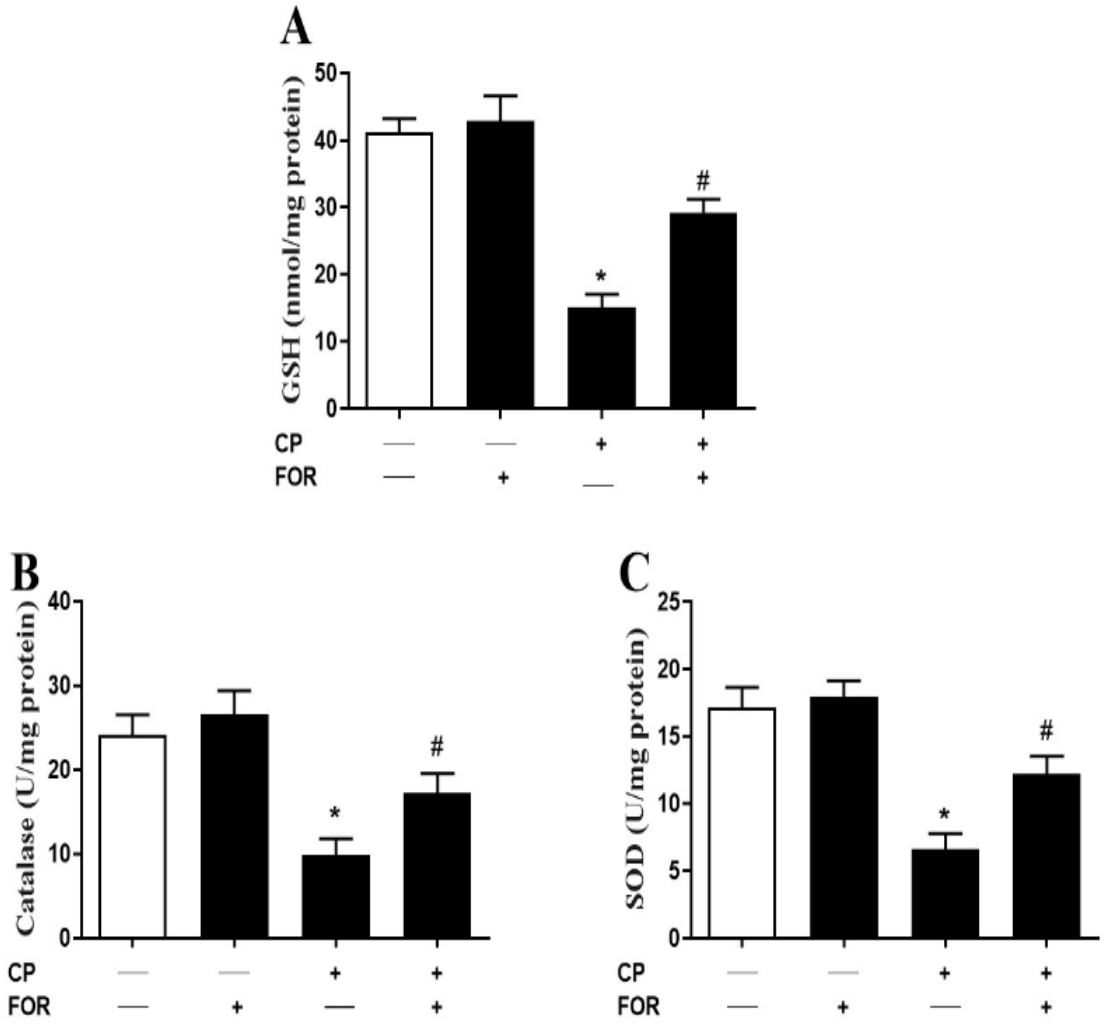

Fig. 3. FOR enhances antioxidants defenses in the kidney

*CP injection caused a significant decrease in (A) GSH content and (B) catalase and (C) SOD activities in the kidney. On the other hand, the FOR treatment of CP-intoxicated rats ameliorated (A) GSH content and (B) catalase and (C) SOD activities in the kidney. Data are shown as Mean ± S.E.M, n=6. * P < 0.05 versus control group. # P < 0.05 versus CP group. FOR: Formononetin; CP: Cyclophosphamide; GSH: Reduced glutathione; SOD: Superoxide dismutase*

3.4 FOR prevents the CP-induced Apoptosis in the kidney

Since CP nephrotoxicity is associated with elevated apoptosis, this study further evaluated the effect of FOR on apoptosis-related proteins in the kidney. CP-induced rats showed significantly increased renal apoptosis as evidenced by decreased Bcl-2 levels and increased Bax and caspase-3 levels in the kidney (Fig. 5A-C). Remarkably, these changes were attenuated when CP-intoxicated rats were pretreated with FOR. FOR alone had no effect on the above-mentioned variables.

4. DISCUSSION

Despite of its importance as anticancer and immunosuppressive drug, CP clinical application is limited because of its nephrotoxicity, which involves several pathways, including oxidative and inflammation, culminating in kidney dysfunction [5,10]. However, in spite of the accumulating knowledge about CP nephro

toxicity, the present preventive strategies are still modest and there is a vital need for development of novel approaches to minimize the CP-induced organ injury. In the present study, we intended to explore the protective effect of FOR against CP-induced nephrotoxicity in rats. Our results showed that FOR can efficiently protect against CP-induced oxidative and inflammation.

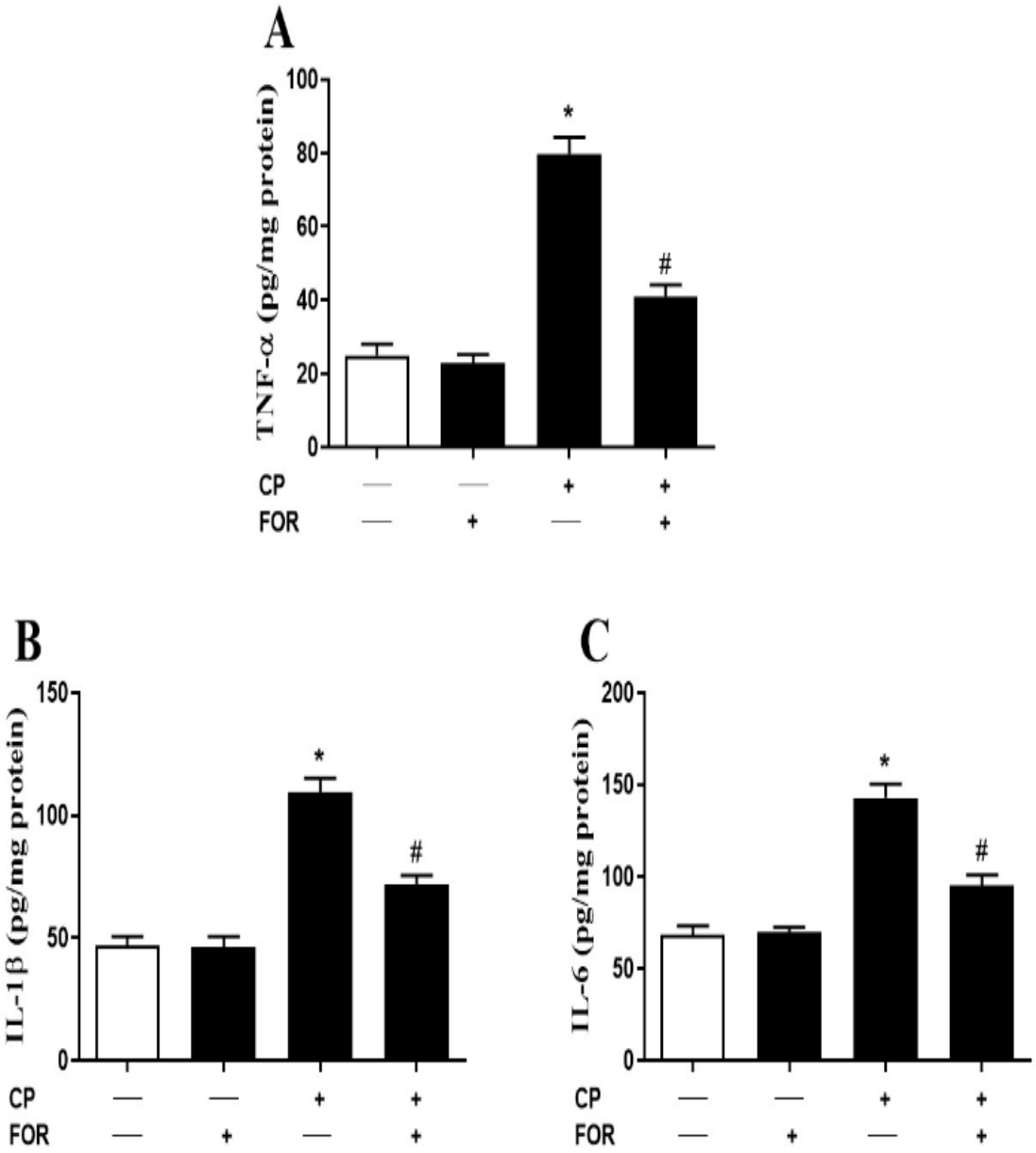

Fig. 4. FOR suppresses the CP -induced inflammation in the kidney

*CP administration induced a significant increase in (A) TNF-α, (B) IL-1β and (C) IL-6 levels in the kidney. Interestingly, The FOR treatment of CP-intoxicated rats attenuated (A) TNF-α, (B) IL-1β and (C) IL-6 levels in the kidney. Data are shown as Mean ± S.E.M, n=6. * P < 0.05 versus control group. # P < 0.05 versus CP group. FOR: Formononetin; CP: Cyclophosphamide; TNF-α: Tumor necrosis factor alpha; IL-1β: Interleukin-1 beta; IL-6: Interleukin 6*

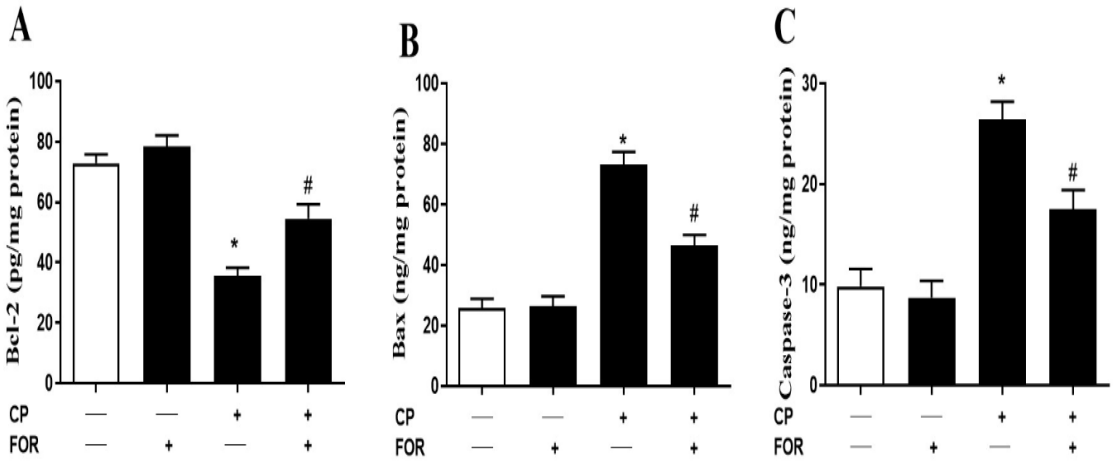

Fig. 5. FOR prevents renal apoptosis in CP-intoxicated rats

*CP administration induced a significant increase in (A) Bcl-2, (B) Bax and (C) caspase-3 levels in the kidney. On contrary, treatment of CP-intoxicated rats with FOR attenuated (A) Bcl-2, (B) Bax and (C) caspase-3 levels in the kidney. Data are shown as Mean ± S.E.M, n=6. * P < 0.05 versus control group. # P < 0.05 versus CP group. FOR: Formononetin; CP: Cyclophosphamide*

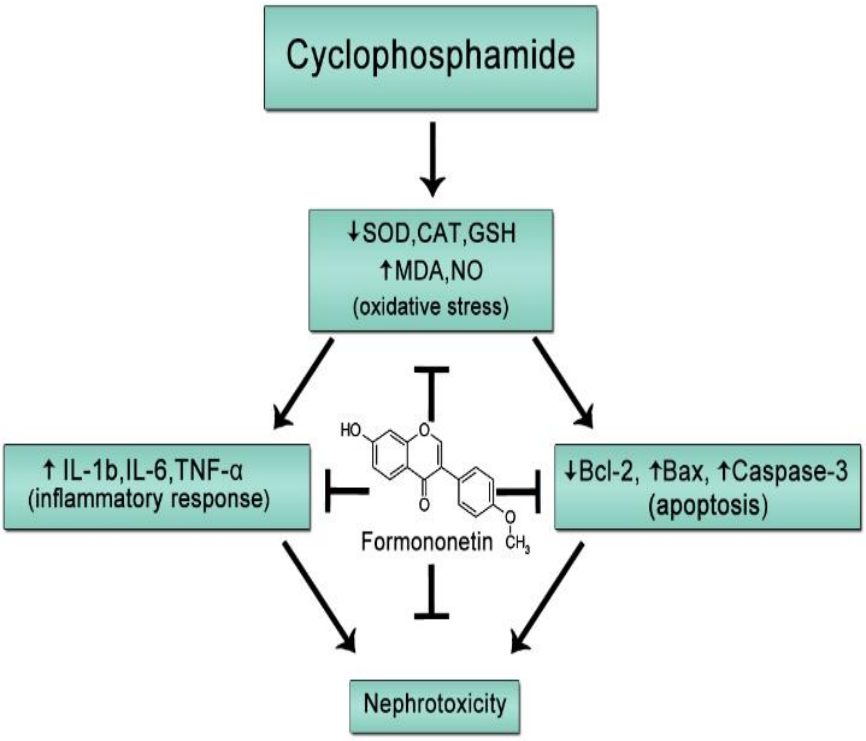

Fig. 6. A schematic diagram illustrating the protective mechanism of FOR against CP nephrotoxicity

FOR boosted antioxidants, suppressed oxidative stress and inhibited inflammation and apoptosis in the kidney of CP-intoxicated rats. MDA: Malondialdehyde; NO: Nitric oxide; GSH: Reduced glutathione; SOD: Superoxide dismutase; TNF-α: Tumor necrosis factor alpha; IL-1β: Interleukin-1 beta; IL-6: Interleukin 6

CP-induced nephrotoxicity was shown by increased creatinine and urea levels in serum. Creatinine is commonly measured as an index of glomerular function [29]. Urea is a resulted from protein breakdown and most of it is excreted through the kidney [30]. High levels of these kidney injury biomarkers indicate deleterious changes in the kidney [29,31]. These results are in agreement with previous studies demonstrating that increased kidney injury biomarkers are the main consequences of CP administration [10,32,33]. Remarkably, FOR pre-treatment afforded renal protection and improved the kidney function in CP-intoxicated rats, indicating a good renoprotective action of FOR. In line with these findings, FOR prevented kidney damage and protected the kidney in rodents model of type 2 diabetes [20], rhabdomyolysis-induced acute kidney injury [18], and cisplatin-induced nephrotoxicity [13].

One of the important molecular mechanisms through which CP injures the kidney is oxidative [5,33]. Indeed, CP has a pro-oxidant nature, and the CP administration leads to production of oxidative stress and also causes an increase in lipid peroxidation and a decrease in the activities of many antioxidant enzymes in different tissues of rodents. In liver, CP is metabolized into two reactive metabolites, acrolein and phosphoramide. These metabolites affect the tissues' antioxidant capacity and lead to production of highly reactive oxygen free radicals [10,34]. Excessive ROS and NO production by CP can cause noticeable cells damage by lipid peroxidation, protein oxidation/nitration, inactivation of enzymes and DNA damage, ultimately leading to cell death [6,11,35]. In agreement with recent studies [5,6,10], the kidney of CP-treated rats showed significant increases in MDA (marker of lipid peroxidation) and NO, coupled with decreased GSH contents and SOD and CAT activities. Lipid peroxidation can cause many problems including alteration of the membrane fluidity and permeability and also might inactivate membrane-bound proteins, this eventually will lead to destruction of the membrane [36]. Importantly, NO can react with superoxide anion, forming the potent cytotoxin peroxynitrite, which affects the mitochondrial and cellular functions, elevates the ROS production and modifies purine and pyrimidine bases, resulting in DNA double-strand breaks and single-strand breaks [37].

FOR has been reported to generate antioxidant actions in many preclinical models that investigates several pathological conditions known to be associated with increased oxidative stress [13,14,16,20]. Based on this, we supposed that the antioxidant efficacy of FOR could be attributed to its protective effect against CP nephrotoxicity. Our results showed that FOR supplementation largely prevented CP-induced increase MDA and NO and enhanced GSH content and antioxidant enzyme activities. In accordance, FOR effectively diminished oxidative stress and boosted cellular antioxidants in kidney of rat models of diabetic nephropathy [20] and cisplatin-induced kidney injury [13]. Importantly, activation of Nrf2, a central regulator of an array of detoxifying and antioxidant defense gene expression [38], might have a role in mediating the antioxidant action of FOR. In the same context, we recently found that FOR prevented CP-induced oxidative tissue injury in the kidney via activation of Nrf2 and induction of HO-1, CAT, and SOD in rats [22]. Thus, FOR prevents CP-induced kidney injury via attenuation of oxidative stress and restoration of antioxidant defenses.

Furthermore, the CP-induced increased ROS generation in the kidney has been found to trigger or potentiate inflammatory mediators production and cause inflammatory responses, such as a rapid increase in the production of proinflammatory cytokines, including IL-6 and TNF-α [39-41]. In experimental renal disease, during the glomerular injury, inflammation has been documented in podocytes and mesangial cells, and also it has been reported in tubular cells as a result of proteinuria and primary tubulointerstitial diseases, including obstruction, and septic or toxic acute kidney injury [42-46]. Indeed, proinflammatory cytokines are implicated in the CP-induced proinflammatory changes in endothelial cells and play a contributory role in the development of intrarenal inflammation [39,40]. Therefore, inhibition of CP-mediated oxidative stress and pro-inflammatory cytokines production can protect against CP-induced nephrotoxicity. Interestingly, treatment of CP-intoxicated rats with FOR prevented kidney damage and reduced the release of proinflammatory cytokines. In further support of the anti-inflammatory effects of this natural isoflavone, FOR blocked IL-1β-induced NF-κB activation and NO production in the rat insulinoma cell line [47]. It has been also reported that FOR suppress streptozotocin (STZ)-induced cognitive impairment by a possible down-regulation of HMGB1/TLR4/NF-κB signaling and NLRP3 inflammasome [48].

To gain more insight into the renoprotective effects of FOR, the present study investigated its effect on caspase-dependent apoptosis. Accumulating evidence indicates that oxidative stress and inflammatory cascade activation may induce apoptotic cell death in the kidney [39,40]. Consistent with several previous studies [6,49], the kidney of CP-injected rats exhibited increased apoptosis, as evidenced by reduced Bcl-2 and elevated Bax and caspase-3 levels. Certainly, CP-mediated apoptosis is believed to be caused by increased ROS production, which in turn sparks the DNA damage, and eventually lead to activation of the mitochondrial apoptotic pathway by enhancing the expression of pro-apoptotic proteins and down-regulating of anti-apoptotic proteins [6,41,49]. Several studies have indicated that the use of antioxidants may have protective effects against CP-induced renal apoptosis [6,10,33]. Herein, pre-treatment with FOR is believed to reverse the CP-induced apoptosis by increasing Bcl-2 levels and decreasing Bax and caspase-3, suggesting that FOR might possess antiapoptotic effects. In support of our findings, FOR prevented apoptosis in rhabdomyolysis-induced kidney injury [18] and cisplatin-induced nephrotoxicity [13] in rodents and attenuated cisplatin-mediated apoptosis in LLC-PK1 cells [50].

5. CONCLUSION

Our findings indicate that the natural isoflavone FOR might has significant therapeutic benefits against the renal complications of CP chemotherapy by having the ability to attenuate oxidative stress, inflammation and apoptosis, and also by promoting antioxidant defenses in the kidney of CP-intoxicated rats (Summarized mechanistic pathways are represented in Fig. 6). Therefore, this study suggests that the protective effects of FOR against CP-induced nephrotoxicity, coupled with its reported antineoplastic properties in various malignancies, are particularly encouraging from a therapeutic point of view. However, further studies are warranted to determine the exact mechanism of FOR action.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

“All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by University of Hafr Al-Batin animal care review committee (G-115-2020)”.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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