18β-Glycyrrhetinic acid protects against methotrexate-induced kidney injury by up-regulating the Nrf2/ARE/HO-1 pathway and endogenous antioxidants

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ABSTRACT

Objectives: 18β-glycyrrhetinic acid (18β-GA) has multiple beneficial and therapeutic effects. However, its protective roles on methotrexate (MTX)-induced renal injury are not well defined. In the present study, we investigated the possible protective effects of 18β-GA against MTX-induced nephrotoxicity in rats.

Materials: 18β-GA (50 and 100 mg/kg) was administered for 7 days either before or after MTX. The rats were decapitated and kidney and serum samples were collected.

Results: MTX-induced renal injury in rats was evidenced by the significant (p < 0.001) increase in circulating kidney function markers and tumor necrosis factor alpha (TNF-α), as well as the histopathological alterations. MTX-induced rats exhibited significantly increased lipid peroxidation (p < 0.05) and nitric oxide (p < 0.001) levels, with concomitant marked (p < 0.001) decline in the antioxidant defenses. 18β-GA, administered either before or after MTX, produced a significant amelioration of circulating kidney function markers, TNF-α, kidney lipid peroxidation, nitric oxide, and antioxidant defenses. In addition, 18β-GA supplementation significantly up-regulated the mRNA abundance of both nuclear factor-erythroid 2-related factor 2 (Nrf2) and hemoxygenase 1 (HO-1) in the kidney of MTX-induced rats.

Conclusions: These results indicate that 18β-GA has a protective effect on MTX-induced nephrotoxicity with possible mechanisms of attenuating oxidative stress and inflammation through up-regulating the Nrf2/ARE signaling. These findings make 18β-GA candidate as a potent agent in preventing MTX-induced kidney injury.

Introduction

The kidney is a vital organ required by the body to perform essential functions such as detoxification, excretion of toxic metabolites and drugs, and the maintenance of homeostasis. Exogenous toxicants and drugs can induce nephrotoxicity in which excretion does not go smoothly. According to Finlay et al., nephrotoxic drugs can be defined as therapeutic agents that have the potential to induce adverse effects on renal function as a result of compromised renal perfusion or direct toxicity. Nephrotoxic drugs often induce inflammation in glomerulus, proximal tubules, and surrounding cellular matrix. The inflammation as well as cytotoxicity occurs due to the increased reactive oxygen species (ROS) production and oxidative stress, the damaged mitochondria in tubules and the disturbed tubular transport system. Studies have demonstrated that approximately 20% of nephrotoxicity is induced by drugs. In this context, the therapeutic applications of chemotherapy have been of limited use due to the induced nephrotoxicity. Methotrexate (MTX), a dihydrofolate reductase inhibitor, is a well-known therapeutic drug for several human malignancies and autoimmune disorders. MTX is commonly applied as part of the first-, second-, or third-line therapy in various autoimmune diseases. Because of its pro-oxidant and nonspecific action, MTX has been reported to induce a variety of adverse effects. Studies have demonstrated that high-dose MTX (HDMTX) is associated with toxicities affecting the kidneys, liver, skin, bone marrow, lungs, and gastrointestinal system. Because 90% of MTX is primarily cleared via the kidneys, severe renal toxicity is one of the factors limiting the use of MTX as an anticancer agent. The study of Widemann and Adamson revealed high incidence of MTX-induced renal toxicity.
within osteosarcoma patients with normal kidney function. In addition, HDMTX has been associated with a broad clinical range of kidney damages varying from subclinical tubulopathy to acute kidney failure.\(^{22}\)

18\(\beta\)-glycyrrhetinic acid (18\(\beta\)-GA) is one of the active ingredients of *Glycyrrhiza glabra* L. (Liquorice).\(^{23}\) Liquorice is widely used for the treatment of various inflammatory diseases, and as a conditioning and flavoring agent.\(^{24}\) Because the natural availability of its 18\(\alpha\) isomer is low, 18\(\beta\)-GA represents a major focus of the current research.\(^{25}\) Previous studies have demonstrated multiple beneficial effects of 18\(\beta\)-GA including hepatoprotective, renoprotective, anti-oxidant, and anti-inflammatory properties.\(^{26-29}\) Recently, we have reported that the hepatoprotective effects of 18\(\beta\)-GA are mediated through up-regulation of the nuclear factor-erythroid 2-related factor 2 (Nrf2).\(^{26}\) Nrf2, a redox-sensitive transcription factor, plays a crucial role in regulating the basal and inducible expressions of several cytoprotective and antioxidant genes.\(^{30,31}\) However, studies on the protective effects of 18\(\beta\)-GA against MTX-induced nephrotoxicity are scarce. The current study was carried out to investigate the possible renoprotective effects of 18\(\beta\)-GA in MTX-induced rats, focusing on its modulatory role on oxidative stress and Nrf2/ARE/HO-1 pathway.

**Materials and methods**

**Chemicals**

MTX was purchased from Shanxi PUDE Pharmaceutical Company (Shanxi, China). 18\(\beta\)-GA, reduced glutathione (GSH), pyrogallol, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 2-(1-naphthylamino)ethylamine dihydrochloride, sulfanilamide, trichloroacetic acid (TCA), thiobarbituric acid (TBA), and 1,1,3,3 tetramethoxypropane were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade and were supplied by standard commercial supplies.

**Experimental animals**

Adult male Wistar rats (8 weeks old) weighing between 120–130 g, obtained from the National Institute of Ophthalmology (Giza, Egypt) were used in the present study. Rats were housed in well-aerated standard cages at normal atmospheric temperature (25 ± 2 °C) and normal 12 h light/dark cycle, and were kept under observation for 1 week for acclimatization. They were supplied daily with standard pellet diet of known composition and given access to water *ad libitum*. All animal procedures were approved by the Institutional Ethics Committee of Beni-Suef University (Egypt).

**Animal grouping and treatments**

Thirty-six rats were used to study the protective effects of 18\(\beta\)-GA against MTX-induced kidney injury. According to the study protocol, animals were divided into six groups as follows (Figure 1):

- **Group I (Control)**: Rats received a single intraperitoneal (i.p.) injection of saline and orally administered the vehicle 0.5% carboxymethylcellulose (CMC) for 7 consecutive days.
- **Group II (MTX)**: Rats received a single i.p. injection of MTX (20 mg/kg)\(^{32}\) and orally administered 0.5% CMC for 7 consecutive days.
- **Group III (50 mg 18\(\beta\)-GA + MTX)**: Rats received 50 mg/kg/day 18\(\beta\)-GA dissolved in 0.5% CMC via oral gavage for 7 consecutive days and a single i.p. injection of MTX (20 mg/kg).
- **Group IV (100 mg 18\(\beta\)-GA + MTX)**: Rats received 100 mg/kg/day 18\(\beta\)-GA dissolved in 0.5% CMC via oral gavage for 7 consecutive days and a single i.p. injection of MTX (20 mg/kg).
- **Group V (MTX + 50 mg 18\(\beta\)-GA)**: Rats received a single i.p. injection of MTX (20 mg/kg) followed by 50 mg/kg/day 18\(\beta\)-GA dissolved in 0.5% CMC via oral gavage for 7 consecutive days.
- **Group VI (MTX + 100 mg 18\(\beta\)-GA)**: Rats received a single i.p. injection of MTX (20 mg/kg) followed by 100 mg/kg/day 18\(\beta\)-GA dissolved in 0.5% CMC via oral gavage for 7 consecutive days.

The dosage of 18\(\beta\)-GA was balanced weekly as indicated by any change in the body weight.

**Samples collection and preparation**

At the end of experiment, rats were killed by cervical decapitation under light ether anesthesia. Blood
samples were collected, left to coagulate and then centrifuged at 3000 rpm for 15 min. The clear non-hemolyzed serum was quickly removed and kept at −20 °C for analysis. The kidneys were quickly removed, rinsed with ice-cold saline, washed, and kept frozen in liquid nitrogen. Frozen samples (10% w/v) were homogenized in cold phosphate-buffered saline (PBS) using Teflon tissue homogenizer (Omni International Inc., Kennesaw, GA), and the homogenates were centrifuged at 3000 rpm for 10 min. The clear homogenates were collected and stored at −80 °C for subsequent assays. Other samples were collected on 10% neutral buffered formalin for histopathological examination or kept at −20 °C for RNA isolation.

Assay of kidney function markers
Serum creatinine, urea, and uric acid levels were determined using reagent kits purchased from Biosystems (Spain), according to the methods of Young,34 Kaplan,35 and Fossati et al.,36 respectively.

Assay of serum tumor necrosis factor alpha (TNF-α)
Serum levels of TNF-α were determined using specific ELISA kits purchased from R&D Systems (Minneapolis, MN), following the manufacturer’s instructions. The concentration of TNF-α was measured spectrophotometrically at 450 nm. Standard curve was constructed by using standard TNF-α, and concentrations of the unknown samples were determined from the standard plot.

Assay of oxidative stress and antioxidant defenses
Lipid peroxidation was assayed in kidney homogenates by measurement of malondialdehyde (MDA) levels according to the method of Preuss et al.37 Nitric oxide (NO) was determined as nitrite levels using Griess reagent. Reduced GSH content and activity of the antioxidant enzymes, superoxide dismutase (SOD), glutathione-S-transferase (G-S-T), and glutathione peroxidase (GPx) were measured according to the methods of Beutler et al.,38 Marklund and Marklund,39 Mannervik and Gutenberg,40 and Matkovics et al.,41 respectively.

RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)
Gene expression analysis of kidney NRF-2 and HO-1 was performed as we previously described.42 Total RNA was isolated from frozen samples using Fermentas RNA isolation kit (Fermentas, USA). Isolated RNA was quantified at 260 nm and RNA integrity was assured by formaldehyde-containing agarose gel electrophoresis. RNA samples with A260/A280 ratios ≥1.7 were selected for reverse transcription. cDNA synthesis was performed with 2 μg RNA using RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, USA). cDNA was amplified using SYBR Green master mix (Fermentas, USA) in a total volume of 20 μl using the primer set listed in Table 1. The PCR reactions were seeded in 96-well plate and the PCR cycles included initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 15 s, annealing at Tm – 5 °C for 30 s, and extension at 72 °C for 30 s. A final step was performed at 60 °C, increased about 0.5 °C every 10 s up to 95 °C. Melting curve analysis was performed to check the specificity of the used primers. The amplification data were analyzed following the 2−ΔΔCt method and the values were normalized to β-actin.

Histopathological study
Histological preparation and examination of kidney samples were performed as we recently reported.7 In brief, the kidney samples were fixed in 10% buffered formalin for at least 24 h and then dehydrated in ascending series of ethanol, cleared in xylene, and embedded in paraffin wax. The blocks were prepared and 4-μm thick sections were cut by a sledge microscope. The sections were deparaffinized, washed, and stained with hematoxylin and eosin (H&E). The stained slides were examined under light microscope.

Statistical analysis
Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA). Results were expressed as mean ± standard error of the mean (SEM) and all statistical comparisons were made by means of the one-way ANOVA test followed by Tukey’s test post hoc analysis. A p value <0.05 was considered significant.

Table 1. Primers pairs used for qRT-PCR.
| Gene  | GenBank accession number | Sequence (5′-3′)          |
|-------|--------------------------|---------------------------|
| NRF2  | NM_031789.2              | F: TTGTAGATGACCATGAGTCGC  |
|       |                          | R: TGTCCTGCTGTATGCTGCTT   |
| HO-1  | NM_012580.2              | F: GAAATGACGGCTGTTGGCCC   |
|       |                          | R: ATGTGCCAGGCTACCTCTTC   |
| β-actin| NM_031144.3             | F: AGGATGACGATGAGTCCGGC   |
|       |                          | R: GGCAGCTCAGTAACAGTGCCG  |
Results

Effect of 18β-GA on kidney function markers

The changes in kidney function markers as a result of MTX administration to rats and oral supplementation of 18β-GA are represented in Figure 2. MTX administration to rats produced a significant (p < 0.001) increase in serum creatinine (Figure 2(A)) and urea (Figure 2(B)) levels when compared with the control rats. Supplementation of both 50 and 100 mg/kg doses of 18β-GA for 7 days, either before or after MTX, significantly (p < 0.001) ameliorated serum creatinine and urea levels when compared with the untreated MTX-induced rats.

Serum uric acid showed a significant (p < 0.001) elevation following MTX administration, as depicted in Figure 2(C). Pretreatment of MTX-administered rats with either dose of 18β-GA significantly (p < 0.05) improved serum uric acid levels when compared with the MTX control group. When supplemented after MTX administration, both doses of 18β-GA produced a significant (p < 0.001) decrease in serum uric acid levels.

Histopathological examination

The microscopic examination of kidney sections of control rats showed normal histological structure of the renal corpuscles and renal tubules (Figure 3(A)). The H&E-stained kidney sections of MTX-induced rats showed dilatation and congestion of renal blood vessel, epithelial cell necrosis, and leukocyte infiltration. The glomeruli were atrophied with diminuted urinary space in some sections and congested in other sections (Figure 3(B–F)). Treatment of the MTX-induced rats with 50 and 100 mg/kg doses of 18β-GA, either before (Figure 3(G,H)) or after (Figure 3(I,J)) MTX administration, produced remarkable amelioration in the kidney tissue and general improvement in the urinary tubules and glomeruli structure.

18β-GA suppresses pro-inflammatory cytokine production in MTX-induced rats

MTX administration to rats produced a significant (p < 0.001) increase in circulating TNF-α levels when
Figure 3. Photomicrographs of H&E-stained kidney sections of (A) control rats, (B–F) MTX-induced rats revealing atrophied glomeruli (arrow), dilatation and congestion of renal blood vessel (arrow head), epithelial cell necrosis and leukocyte infiltration (dashed arrow), (G) MTX-administered rats pretreated with 50 mg/kg 18β-GA, (H) MTX-administered rats pretreated with 100 mg/kg 18β-GA, (I) MTX-administered rats treated with 50 mg/kg 18β-GA and (J) MTX-administered rats treated with 100 mg/kg 18β-GA, showing noticeable amelioration in the kidney tissue and general improvement in the urinary tubules and glomeruli structure.
compared with the control rats, as depicted in Figure 4.

18β-GA at either dose significantly \( p < 0.001 \) decreased serum TNF-α when supplemented prior to MTX administration. Similarly, 18β-GA significantly decreased circulating levels of TNF-α in MTX-administered rats when supplemented at either 50 \( p < 0.01 \) or 100 mg/kg \( p < 0.001 \) following MTX.

**18β-GA attenuates oxidative stress in kidney of MTX-induced rats**

Data summarized in Figure 5 show the effect of 18β-GA on lipid peroxidation and NO levels in the kidney of MTX-induced rats. MTX administration produced a significant \( p < 0.05 \) increase in kidney lipid peroxidation marker MDA when compared with the corresponding control rats (Figure 5(A)). Pretreatment of the MTX-induced rats with either 50 or 100 mg/kg 18β-GA dose significantly \( p < 0.001 \) decreased kidney MDA levels. Similarly, both 18β-GA doses administered after MTX markedly \( p < 0.001 \) decreased kidney lipid peroxidation levels.

Similar to lipid peroxidation, kidney NO levels showed a significant \( p < 0.001 \) increase in MTX-induced rats when compared with the corresponding control group (Figure 5(B)). 18β-GA produced a significant decrease in kidney NO levels when administered for 7 days before MTX at both the 50 \( p < 0.05 \) and 100 mg/kg \( p < 0.01 \) doses. The 50 as well as 100 mg/kg 18β-GA doses supplemented for 7 days after MTX administration markedly \( p < 0.01 \) decreased NO in the kidney of MTX-induced rats.

The effects of 18β-GA on GSH and activity of SOD, GPx, and G-S-T in kidney of MTX-induced rats are represented in Figure 6. GSH exhibited a significant \( p < 0.001 \) decline in kidney of MTX-induced rats when compared with the corresponding control group (Figure 6(A)). Pretreatment of the MTX-administered rats with 50 mg/kg 18β-GA dose significantly \( p < 0.05 \) prevented MTX-induced GSH decline in kidney of rats. Pretreatment with the 100 mg/kg 18β-GA markedly \( p < 0.01 \) rejuvenated kidney GSH content in MTX-administered rats. Oral supplementation of 50 or 100 mg/kg 18β-GA for 7 days after MTX administration produced marked \( p < 0.05 \) alleviation in kidney GSH content.

MTX-administered rats exhibited marked decrease \( p < 0.001 \) in kidney SOD (Figure 6(B)), GPx (Figure 6(C)), and G-S-T (Figure 6(D)) activity when compared with the control rats. Pretreatment of the MTX-induced rats with 50 mg/kg dose of 18β-GA significantly improved the activity of SOD \( p < 0.05 \), GPx \( p < 0.001 \), and G-S-T \( p < 0.05 \). The same dose administered after

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**Figure 4.** Effect of 18β-GA on serum TNF-α levels in MTX-induced rats. Data are expressed as mean ± SEM \( (N = 6) \). ***\( p < 0.001 \) versus Control, and ###\( p < 0.001 \) versus MTX. 18β-GA: 18β-glycyrrhetinic acid; MTX: methotrexate; TNF-α: tumor necrosis factor alpha.

**Figure 5.** Effect of 18β-GA on (A) lipid peroxidation and (B) nitric oxide levels in kidney of MTX-induced rats. Data are expressed as mean ± SEM \( (N = 6) \). *\( p < 0.05 \) and ***\( p < 0.001 \) versus Control, and #\( p < 0.05 \), ###\( p < 0.01 \) and ####\( p < 0.001 \) versus MTX. 18β-GA: 18β-glycyrrhetinic acid; MTX: methotrexate; MDA: malondialdehyde; NO: nitric oxide.
MTX exerted significant ($p < 0.05$) ameliorative effect on SOD, GPx, and G-S-T. The 100 mg/kg 18β-GA dose supplemented for 7 days before MTX administration produced significant amelioration in the activity of SOD ($p < 0.001$), GPx ($p < 0.001$), and G-S-T ($p < 0.05$). More or less similar, the same dose supplemented for 7 days after MTX administration significantly improved kidney SOD ($p < 0.001$), GPx ($p < 0.05$), and G-S-T activity ($p < 0.05$) when compared with the MTX group of rats.

Figure 6. Effect of 18β-GA on (A) reduced glutathione, (B) SOD, (C) GPx, and (D) G-S-T in kidney of MTX-induced rats. Data are expressed as mean ± SEM ($N = 6$). ***$p < 0.001$ versus Control, and #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ versus MTX. 18β-GA: 18β-glycyrrhetic acid; MTX: methotrexate; GSH: reduced glutathione; SOD: superoxide dismutase; GPx: glutathione peroxidase; G-S-T: glutathione-S-transferase.

Figure 7. Effect of 18β-GA on mRNA expression levels of (A) Nrf2 and (B) HO-1 in kidney of MTX-induced rats. Data are expressed as mean ± SEM ($N = 6$). ***$p < 0.001$ versus Control, and #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ versus MTX. 18β-GA: 18β-glycyrrhetic acid; MTX: methotrexate; Nrf2: nuclear factor erythroid 2-related factor 2; HO-1: heme oxygenase 1.
18β-GA up-regulates the Nrf2/ARE/HO-1 signaling pathway in kidney of MTX-induced rats

To study the effect of 18β-GA on Nrf2/ARE/HO-1 in kidney of MTX-induced rats, we determined mRNA expression of Nrf2 and HO-1 using qRT-PCR.

As represented in Figure 7(A), Nrf2 mRNA expression in the kidney of MTX-induced rats showed a significant \( (p < 0.001) \) down-regulation when compared with the corresponding control rats. Pretreatment of the MTX-administered rats with either 50 or 100 mg/kg 18β-GA significantly \( (p < 0.01) \) up-regulated Nrf2 mRNA expression when compared with the MTX group of rats. 18β-GA supplemented following MTX administration produced a significant increase in Nrf2 mRNA abundance at both 50 \( (p < 0.05) \) and 100 mg/kg \( (p < 0.01) \) doses.

HO-1 mRNA expression showed a significant \( (p < 0.001) \) down-regulation in MTX-administered rats when compared with the control group (Figure 7(B)). The 50 mg/kg 18β-GA dose produced a significant increase in HO-1 mRNA expression when supplemented either before \( (p < 0.01) \) or after \( (p < 0.05) \) MTX. Oral supplementation of 100 mg/kg dose of 18β-GA, either before or after MTX, produced a significant \( (p < 0.01) \) up-regulation of HO-1 mRNA expression.

Discussion

In this investigation, we evaluated the protective effects of 18β-GA on MTX-induced nephrotoxicity in rats. We found that 18β-GA, administered before or after MTX, protected against kidney injury through up-regulating the Nrf2/ARE/HO-1 pathway and attenuating inflammation and oxidative stress.

Here, MTX induced renal damage as indicated from the significant increase in creatinine, urea and uric acid levels. These measurements are often used as reliable markers of renal damage,\(^{43}\) and indicate loss of a majority of kidney functions.\(^{44}\) In agreement with our findings, several studies have demonstrated MTX-induced renal toxicity with elevated serum creatinine and urea both in patients and animal models.\(^{18,45,46}\) These biochemical findings were further supported by the histopathological data which showed dilatation and congestion of renal blood vessels, epithelial cell necrosis, leucocyte infiltration, and some atrophied glomeruli with diminished urinary space. Smeland et al.\(^{47}\) reported that this toxicity occurs due to the precipitation of MTX or its metabolites in the renal tubules causing obstruction and diminution of renal clearance. In addition, the study of Widemann and Adamson\(^{21}\) has demonstrated MTX-induced acute kidney injury (AKI) which results from its crystallization in the renal tubules when administered intravenously.

18β-GA, supplemented for 7 days either before or after MTX, significantly improved circulating creatinine, urea, and uric acid levels, and remarkably attenuated the induced histological alterations in the kidney. Accordingly, Wu et al.\(^{28}\) showed the protective effect of 18β-GA against cisplatin-induced nephrotoxicity in BALB/c mice. More recently, Ma et al.\(^{29}\) demonstrated the anti-apoptotic effect of 18β-GA in renal tubular epithelial cells.

MTX-induced rats showed a significant increase in circulating TNF-α levels. TNF-α is a pro-inflammatory cytokine produced in response to MTX\(^{18}\) as well as other nephrotoxicants.\(^{48,49}\) In renal disease, cytokines up-regulate endothelial cell adhesion molecules and chemokines which provoke renal inflammatory cell infiltration.\(^{50,51}\) In addition, cytokines can increase activation of nuclear factor kappa-B (NF-κB), promoting a pro-inflammatory phenotype.\(^{52,53}\) The expression and/or activation of NF-κB have been demonstrated in the kidney from AKI patients and experimental animals.\(^{18}\) In addition, pro-inflammatory cytokines can activate neutrophils and macrophages resulting in increased ROS production.\(^{51}\) The study of Coskun et al.\(^{54}\) showed that TNF-α induces neutrophil-mediated tissue injury. In experimental animals, MTX has been reported to induce the accumulation of neutrophils in the kidney.\(^{55}\) Oral supplementation of 18β-GA significantly decreased the circulating levels of TNF-α in MTX-induced rats. These findings could be attributed to the suppressed expression of NF-κB following treatment with 18β-GA as recently showed.\(^{26,28}\)

Oxidative stress is a hallmark of MTX-induced nephrotoxicity.\(^{16,56}\) In the present study, MTX administration produced a marked increase in lipid peroxidation and NO levels in the kidney of rats, revealing oxidative stress. Consequently, GSH content showed a significant decline in kidney of MTX-induced rats. GSH acts as a potent antioxidant by forming S-conjugates with products of lipid peroxidation.\(^{57}\) Activity of the antioxidant enzymes SOD, GPx, and G-S-T was decreased in kidneys of the MTX-induced rats. These antioxidant defense enzymes play a key role in protecting against the deleterious effects of ROS.\(^{58}\) Decreased activity of antioxidant enzymes and depletion of GSH lead to mitochondrial dysfunction which causes necrosis and impaired renal function.\(^{59}\)

MTX has been reported to repress the cellular antioxidant defenses, thereby intensifying oxidative stress in many organs.\(^{60}\) Kilic et al.\(^{61}\) demonstrated that MTX increases the amount of hydrogen peroxide and stimulates neutrophils, leading to the release of other ROS.
It hampers the remethylation of homocysteine leading to elevated homocysteine levels, causing ROS generation.\(^{18}\) ROS induce cellular injury, protein damage, DNA fragmentation, and lipid peroxidation.\(^{62}\) The elevated ROS play a key role in mediating apoptosis which have been implicated in MTX-induced tissue damage.\(^{63}\) In addition, NO has been reported to play a key role in acute renal failure.\(^{64}\) It reacts with superoxide radical forming the cytotoxic versatile and potent oxidant peroxynitrite.\(^{65}\)

Oral supplementation of 18β-GA markedly decreased kidney MDA and NO levels, and enhanced both the enzymatic and non-enzymatic antioxidant defenses, demonstrating the potent free radical scavenging and antioxidant efficacies of 18β-GA. A previous study conducted by Kao et al.\(^{66}\) indicated that 18β-GA has excellent antioxidant characteristics that it promoted antioxidant enzymes in neuronal cells and inhibited ROS-induced oxidative damage and apoptosis. In addition, 18β-GA protected BALB/c mice against cisplatin-induced oxidative stress by preventing GSH depletion.\(^{26}\) Recently, we have reported that the protective effect of 18β-GA against cyclophosphamide-induced hepatotoxicity was mediated through its potent antioxidant and radical scavenging properties.\(^{26}\) Therefore, we assume that prevention of GSH decline and potentiation of the antioxidant enzymes mediates the protective mechanism of 18β-GA against MTX-induced nephrotoxicity. The decreased NO levels could be attributed to the ability of 18β-GA to suppress the expression of inducible nitric oxide synthase (iNOS), as we recently reported.\(^{26}\) iNOS-induced NO production may mediate lipid peroxidation.\(^{67}\) Therefore, decreased NO levels by 18β-GA could mediate, at least in part, its suppressive effect on the generation of lipid peroxides.

The enhanced efficacy of antioxidant defense system could be directly connected to the ability of 18β-GA to positively regulate the Nrf2/ARE/HO-1 signaling pathway. MTX administration produced a significant down-regulation of Nrf2 and HO-1 mRNA abundance, an effect that was potentially reversed by 18β-GA supplemented before or after MTX. Nrf2 plays a crucial role in regulating the basal and inducible expressions of several cytoprotective and antioxidant genes, which can counteract oxidative stress.\(^{30,31}\) These include genes encoding antioxidant enzymes such as SOD, CAT, HO-1, GPx, and G-S-T.\(^{68,69}\)

Therefore, the observed induction of antioxidant enzymes by 18β-GA may be explained, at least in part, by the functional activation of Nrf2/HO-1 signaling pathway in kidney of MTX-induced rats. In agreement with our findings, Wu et al.\(^{28}\) reported the positive effect of 18β-GA on Nrf2 expression in kidney of cisplatin-intoxicated mice. We have demonstrated the ability of 18β-GA to up-regulate both gene and protein expression of Nrf2 in the liver of cyclophosphamide-induced rats.\(^{26}\) HO-1 expression has also been demonstrated to be up-regulated by 18β-GA to eliminate ROS
formation.70 Furthermore, the renoprotective role of Nrf2 is supported by the finding that streptozotocin-induced diabetic rats supplemented with dietary Nrf2 activators showed limited albuminuria and renal oxidative damage.71 However, the free radical scavenging ability of 18β-GA should be considered as a mechanism priming the antioxidant defenses inducing effect in response to MTX.

Moreover, up-regulation of Nrf2 expression may exert a role in the anti-inflammatory effect of 18β-GA in MTX-induced rats. Nrf2/ARE is one of the pathways implicated in the regulation of inflammation.72 In this context, previous experiments have demonstrated several anti-inflammatory phytochemicals which activate the Nrf2/ARE pathway and suppress NF-κB.73 The positive regulatory effect of 18β-GA on Nrf2/ARE pathway has also been associated with repressed NF-κB and iNOS expression in experimental rats.26,28

In conclusion, the present study confers new information on the renoprotective effect of 18β-GA on MTX-induced injury in rats. 18β-GA attenuated oxidative and nitrosative stresses mitigated inflammation and prevented MTX-induced histopathological alteration in the kidney of rats. These protective effects appear to depend, at least in part, on the up-regulation of Nrf2 and subsequent enhancement of the antioxidant defenses (summarized mechanistic pathways are represented in Figure 8).

Disclosure statement
The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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