

Harmine Attenuates Methotrexate-Induced Nephrotoxicity in Mice through Suppression of Oxidative Stress

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ABSTRACT

Although methotrexate is widely used in clinical practice, its therapeutic application is frequently restricted due to adverse effects, particularly nephrotoxicity. Oxidative stress is recognized as a primary mechanism underlying methotrexate-induced renal injury. Harmine, a naturally occurring plant-derived compound, exhibits antioxidant and anti-inflammatory activities. This study aimed to investigate the protective effects of harmine against methotrexate-induced kidney toxicity. Mice were randomly assigned to six experimental groups: control (saline), methotrexate (20 mg/kg), harmine (20 mg/kg), and methotrexate (20 mg/kg) combined with harmine at doses of 5, 10, or 20 mg/kg. All treatments were administered intraperitoneally over a 14-day period. At the end of the experiment, blood and kidney tissues were collected for further analysis. Histopathological evaluation was performed using hematoxylin–eosin (H&E) staining, while molecular and biochemical assessments were conducted using qRT-PCR and standard biochemical assays. Administration of methotrexate resulted in a significant elevation of serum creatinine and blood urea nitrogen levels, whereas treatment with harmine at doses of 10 and 20 mg/kg significantly attenuated these changes. Harmine also improved both the number and diameter of glomeruli in methotrexate-treated mice. In addition, methotrexate markedly increased renal malondialdehyde and nitric oxide levels and reduced total antioxidant capacity and superoxide dismutase activity. Harmine treatment effectively reduced oxidative stress markers and restored antioxidant defense systems. Furthermore, harmine downregulated the methotrexate-induced upregulation of Ho-1, Nqo1, Trx1, and Nrf2 mRNA expression. Histological abnormalities caused by methotrexate were also substantially alleviated by harmine administration. These findings indicate that harmine exerts a protective effect against methotrexate-induced nephrotoxicity, likely through the suppression of oxidative stress and enhancement of antioxidant mechanisms.

Keywords: Oxidative stress, Toxicity, Methotrexate, Harmine

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Introduction

Nephrotoxicity from medications remains a prevalent complication and a leading factor in acute kidney injury [1]. Methotrexate, a small-molecule chemotherapy agent, is commonly prescribed for treating various cancers and autoimmune disorders [2-6]. Its widespread use is often hampered by significant side effects, with kidney damage being particularly prominent [3, 7-9]. Given that the drug is primarily cleared by the kidneys in its unchanged form [10], especially high-dose regimens can trigger acute renal impairment [2]. The kidney injury associated with methotrexate is primarily attributed to two key processes [11]. One involves the formation of crystals from methotrexate and its metabolites inside the renal tubules, causing blockage, reduced drug clearance, sustained high blood levels, and amplified toxic effects [12]. The other pathway entails direct harm to tubular cells mediated by heightened oxidative stress, where methotrexate triggers overproduction of reactive oxygen species (ROS) in kidney cells [13].

Cells possess various built-in antioxidant mechanisms to manage oxidative balance [14]. Research has consistently shown that methotrexate impairs renal antioxidant defenses, leading to increased lipid peroxidation, higher malondialdehyde (MDA) concentrations, elevated myeloperoxidase (MPO) activity, and enhanced nitric oxide (NO) production in kidney tissue. Concurrently, it suppresses catalase, glutathione (GSH), and superoxide dismutase (SOD) activities in both circulation and renal parenchyma [13, 15, 16]. In addition to oxidative damage, dysregulation of inflammatory cytokines and influx of neutrophils play critical roles in exacerbating methotrexate-related kidney injury [16, 17].

Efforts are ongoing to improve the safety profile of methotrexate by identifying adjunctive agents that can counteract its renal toxicity. Particular emphasis has been placed on plant-derived compounds known for their antioxidant and anti-inflammatory potentials as a means to lessen these adverse effects. Notable examples include curcumin, gallic acid, ferulic acid, caffeic acid phenethyl ester, thymoquinone, vincamine, and silymarin [9, 15, 17-22]. Harmine, a β -carboline alkaloid (chemically 7-methoxy-1-methyl-9H-pyrido[3,4-b]indole) isolated from plants like *Peganum harmala* L., displays diverse bioactivities, including antimicrobial, antioxidant, and anticancer properties [23]. Prior work has highlighted harmine's ability to attenuate nicotine-evoked renal damage by reducing MDA, creatinine, and NO concentrations [24]. It has also exhibited protective actions against lipopolysaccharide-induced acute renal injury through mitigation of oxidative stress [25]. Motivated by these observations, this investigation examined whether harmine could safeguard against methotrexate-triggered kidney toxicity and clarified the role of oxidative stress components in this process.

Materials and Methods

Ethical considerations

This study was approved by the Ethics Committee at Kermanshah University of Medical Sciences (approval code: IR.KUMS.REC.1398.1171). All procedures involving animals adhered to the principles outlined in the Helsinki declaration and institutional ethical standards.

Chemicals

Pure harmine powder (7-methoxy-1-methyl-9H-pyrido[3,4-b]indole; CAS No: 442-51-3) was acquired from Sigma. Dosing regimens were informed by earlier publications confirming its antioxidant and anti-inflammatory efficacy *in vivo* [25, 26]. Xylazine was obtained from Alfasan Co., Netherlands. Commercial kits from Pars Azmoon (Iran) were used to quantify serum creatinine and blood urea nitrogen (BUN). Total antioxidant capacity was measured employing RANDOX TAS reagents (UK). Nitric oxide was assayed via the Promega Griess Reagent System. RNA isolation utilized Invitrogen's Trizol reagent (USA), while cDNA was generated with TaKaRa Bio's PrimeScript First Strand cDNA Synthesis Kit (Japan). Real-time gene amplification relied on TaKaRa's SYBR Green Master Mix (Japan).

Animals and experimental protocol

A total of 42 adult male Balb/c mice (27–30 g) were maintained under standard conditions: 12-hour light/dark cycles, ambient temperature of 23 ± 2 °C, and *ad libitum* access to standard chow and water.

Mice were allocated randomly into six groups of seven animals each: (1) control (normal saline vehicle); (2) methotrexate alone (20 mg/kg) with saline; (3) harmine alone (20 mg/kg); and (4–6) methotrexate (20 mg/kg) combined with harmine at 5, 10, or 20 mg/kg.

All injections were intraperitoneal and given daily over 14 consecutive days. Drugs were dissolved fresh in normal saline. On the 15th day (24 hours post-final injection), mice were anesthetized using ketamine (70 mg/kg) plus xylazine (10 mg/kg). Blood was sampled via the abdominal aorta, followed by centrifugation (3,000 rpm, 15 min, 4 °C) for serum isolation. Kidneys were promptly removed; portions were allocated for histopathology, with the rest snap-frozen in liquid nitrogen and archived at -80 °C for molecular analyses.

Histopathological analysis

Excised kidneys were immersed in 10% neutral buffered formalin for 24 hours. Post-fixation, samples were halved longitudinally, processed into paraffin blocks, and cut into 5–7 μ m sections. Slides were subjected to standard hematoxylin-eosin staining and viewed under an Olympus CH3 light microscope (Japan). Quantitative assessment

involved measuring glomerular diameters in 100 randomly selected glomeruli per sample using associated DP2-BSW software, alongside counting glomerular density in multiple fields.

Biochemical assays

Creatinine and blood BUN

Standard commercial kits were employed to determine serum creatinine and BUN, following provided protocols.

Oxidative stress and antioxidant markers

Superoxide dismutase (SOD) activity in serum was quantified per Nishikimi *et al.* [27], while malondialdehyde (MDA) followed Ohkawa *et al.* [28]. Total antioxidant capacity was assessed using the designated kit, and nitric oxide levels were determined with Griess reagent, aligned with Giustarini *et al.* [29].

Quantitative transcription polymerase chain reaction (qRT-PCR)

Renal tissue was homogenized, and total RNA extracted via Trizol. Reverse transcription to cDNA used the PrimeScript kit. Amplification and quantification of target transcripts were performed on a Corbett Rotor-Gene 6000 instrument with SYBR Green chemistry. Expression data were normalized to β -actin and reported as fold changes versus the control group. Specific primer details are provided in **Table 1**.

Table 1. Primers used for qRT-PCR analysis

Primer	Sequence
Nrf2	Forward: 5'-CAGCATGATGGACTTGGA-3' Backward: 5'-TGAGACACTGGTCACACT-3'
Ho-1	Forward: 5'-CCTTCCCGAACATCGACAGCC-3' Backward: 5'-GCAGCTCCTCAAACAGCTCAA-3'
Trx1	Forward: 5'-CCCTTCTTCCATTCCCTCT-3' Backward: 5'-TCCACATCCACTTCAAGGAAC-3'
Nqo1	Forward: 5'-AAGGATGGAAGAAACGCCTGGAGA-3' Backward: 5'-GGCCACAGAAAGGCCAAATTCT-3'

Statistical analysis

All statistical evaluations were carried out using SPSS software (version 19.0; SPSS, Chicago, IL, USA). Differences among experimental groups were analyzed by one-way analysis of variance (ANOVA), followed by Tukey’s multiple comparison test. Results are reported as mean \pm standard error of the mean (SEM). A p-value of less than 0.05 was considered indicative of statistical significance.

Results and Discussion

The present study provides evidence that harmine exerts a protective effect against methotrexate-induced renal injury in mice, primarily through enhancement of antioxidant capacity and regulation of oxidative stress-responsive genes. Methotrexate is an effective antineoplastic agent, but its clinical application remains limited due to organ toxicity, particularly nephrotoxicity [11]. Previous studies have demonstrated that various phytochemicals possessing intrinsic antioxidant properties can attenuate methotrexate-induced renal damage by restoring redox balance and reinforcing endogenous antioxidant defenses [9, 17, 19, 20, 22]. Harmine, the compound investigated in this study, is a naturally occurring alkaloid with well-documented biological activities, including antioxidant and nephroprotective effects [25, 26]. Its protective roles have been previously reported in models of dioxin-induced liver injury [30] and lipopolysaccharide (LPS)-induced acute kidney damage [25]. However, to date, its role in methotrexate-associated nephrotoxicity had not been evaluated. Accordingly, this study focused on determining whether harmine could alleviate methotrexate-induced renal injury in mice.

Renal dysfunction caused by methotrexate is commonly associated with elevations in serum creatinine and blood urea nitrogen (BUN), as well as clinical manifestations such as hematuria and uremia [9, 31].

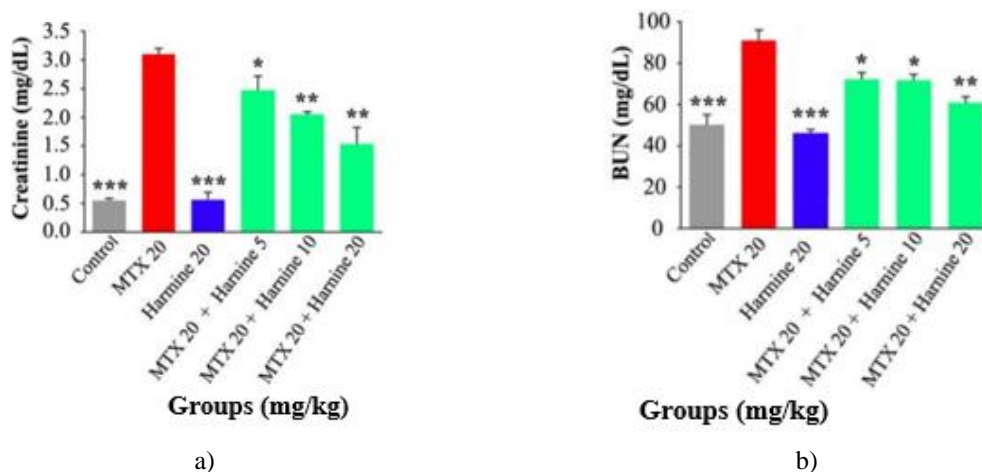


Figure 1. Effects of harmine (5, 10, and 20 mg/kg) and methotrexate (20 mg/kg) on kidney function indices. Values are expressed as mean \pm SEM (n = 7). *p < 0.05, **p < 0.01, ***p < 0.001 compared with the methotrexate group. BUN: blood urea nitrogen; MTX 20: methotrexate (20 mg/kg).

Consistent with these reports, methotrexate administration in the current study resulted in a marked increase in serum creatinine (**Figure 1a**) and BUN concentrations (**Figure 1b**) relative to the control group (p < 0.001). In contrast, co-administration of harmine at doses of 10 and 20 mg/kg significantly reduced both parameters compared with the methotrexate-only group (p < 0.05 and p < 0.01, respectively). These biochemical improvements were supported by histopathological findings. Kidney sections from control animals exhibited normal glomerular and tubular morphology (**Figure 2a**). In methotrexate-treated mice, extensive pathological alterations were observed, including tubular dilation, epithelial cell detachment, vascular congestion, glomerular fibrosis, and intracellular vacuolation (**Figure 2b**). Administration of harmine alone (20 mg/kg) did not induce pathological changes and preserved renal architecture similar to that of controls (**Figure 2c**). Moreover, concurrent treatment with harmine (20 mg/kg) substantially reduced methotrexate-induced structural damage (**Figure 2d**). Quantitative histological data are presented in **Table 2**.

Morphometric evaluation further revealed that methotrexate exposure significantly decreased both the number of glomeruli (p < 0.001) and their diameter (p < 0.01). These deleterious effects were significantly reversed following harmine treatment, indicating preservation of glomerular integrity in methotrexate-challenged mice (**Figures 2e and 2f**).

Oxidative stress has been widely recognized as a central mechanism underlying methotrexate-induced nephrotoxicity, often occurring in parallel with inflammatory processes [8, 9, 13, 22]. Excessive production of reactive oxygen species (ROS), depletion of antioxidant reserves, and enhanced lipid peroxidation are characteristic features of methotrexate-induced injury in renal and other organs [8, 9]. Several mechanisms have been proposed to explain methotrexate-induced ROS generation, including neutrophil infiltration and activation [32], mitochondrial dysfunction [33], and increased NADPH oxidase activity [34]. Elevated ROS levels can also promote the activation of inflammatory signaling pathways, leading to increased expression of nuclear factor-kappaB (NF- κ B), tumor necrosis factor-alpha (TNF α), and interleukins such as IL-1 β and IL-10, which play key roles in methotrexate-associated renal damage [21, 35].

Strategies aimed at reducing oxidative burden and reinforcing endogenous antioxidant defenses have been shown to be effective in mitigating methotrexate-induced kidney injury [19, 21]. In this context, harmine was evaluated for its antioxidant efficacy. Total antioxidant capacity (TAC), malondialdehyde (MDA), and superoxide dismutase (SOD) activity were measured to assess oxidative stress status and the protective role of harmine (**Figure 3**). Methotrexate treatment resulted in a pronounced reduction in TAC compared with the control group (p < 0.001), whereas administration of harmine at all tested doses (5, 10, and 20 mg/kg) significantly restored TAC levels (**Figure 3a**).

In parallel, renal MDA levels were markedly elevated following methotrexate exposure (p < 0.001), indicating increased lipid peroxidation. Treatment with harmine at doses of 10 or 20 mg/kg significantly reduced MDA concentrations, demonstrating its ability to attenuate oxidative membrane damage (p < 0.05); (**Figure 3b**). These

findings are in agreement with earlier studies reporting that harmine reduces oxidative stress markers, including MDA, creatinine, BUN, and nitric oxide, in nicotine-induced renal injury [24]. Additionally, methotrexate administration significantly suppressed SOD activity after 14 days compared with the control group ($p < 0.001$). Notably, harmine treatment at a dose of 20 mg/kg significantly restored SOD activity ($p < 0.01$); (**Figure 3c**). Similar observations have been reported in other experimental models, where methotrexate increased oxidative stress markers such as MDA while reducing antioxidant enzyme activity [22]. Furthermore, Niu *et al.* demonstrated that methotrexate decreased glutathione levels and SOD activity in hepatic, renal, and cardiac tissues, whereas harmine pre-treatment markedly protected against LPS-induced acute kidney injury by reducing MDA and MPO formation and enhancing SOD and GSH levels [25].

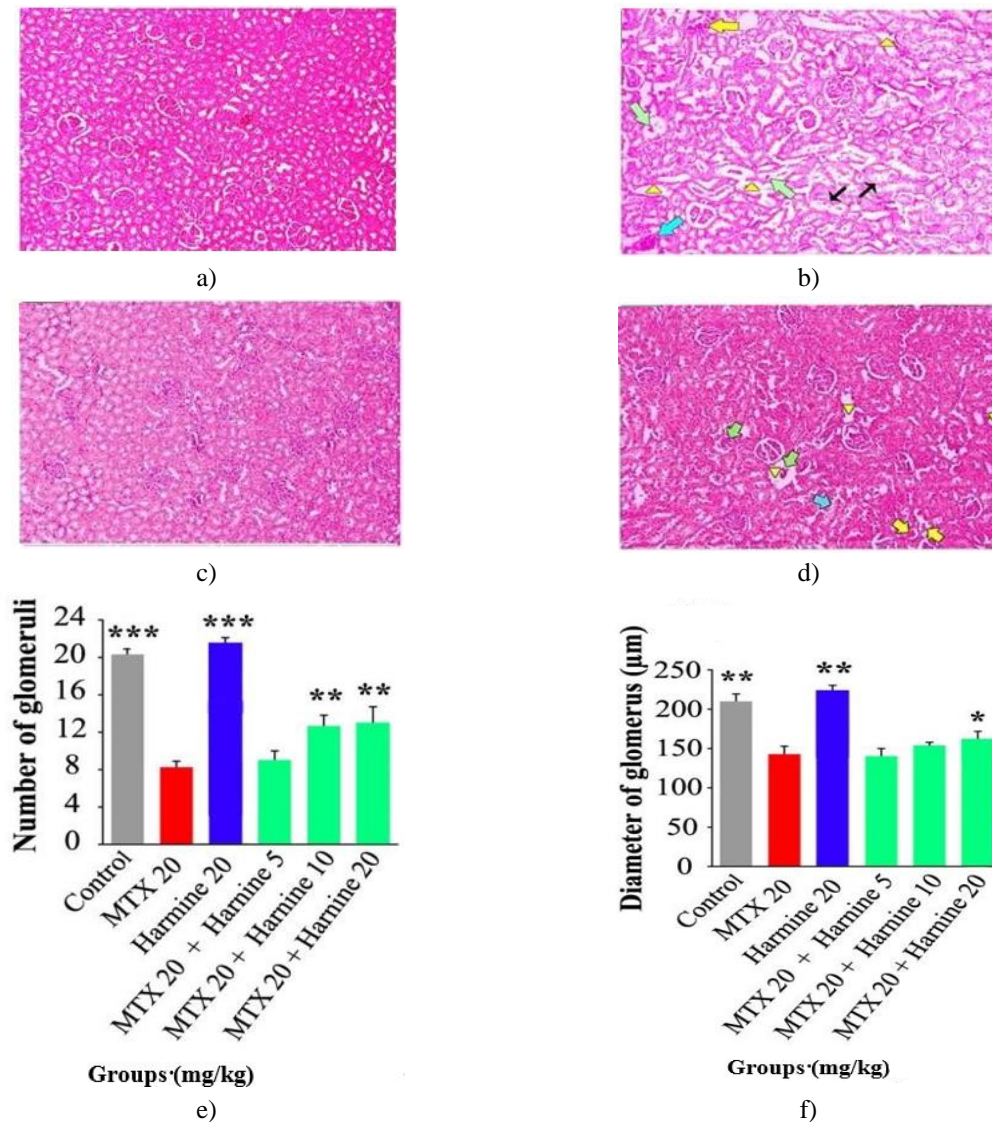


Figure 2. Histopathological effects of harmine (5, 10, and 20 mg/kg) and methotrexate (20 mg/kg) on renal tissue in mice. Panels a–d show hematoxylin and eosin (H&E)–stained kidney sections: (a) control group displaying normal renal morphology; (b) methotrexate-treated group exhibiting proteinaceous casts (yellow arrow), tubular epithelial cell detachment (green arrow), intracellular vacuolization (black arrow), and glomerular fibrosis (blue arrow). The yellow triangle indicates dilation of renal tubules; (c) harmine-treated group (20 mg/kg); (d) methotrexate (20 mg/kg) co-administered with harmine (20 mg/kg). Panels (e) and (f) represent quantitative analyses of glomerular number and glomerular diameter (μm), respectively. Data are expressed as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the methotrexate group. MTX 20: methotrexate (20 mg/kg); harmine 5, 10, and 20: harmine administered at doses of 5, 10, and 20 mg/kg, respectively.

Mice exposed to methotrexate exhibited a pronounced elevation in nitric oxide (NO) levels compared with the control group ($p < 0.001$). Treatment with harmine reduced NO concentrations in a dose-dependent manner, with a statistically significant decrease observed at the 20 mg/kg dose ($p < 0.001$); (**Figure 3d**). Nitric oxide has been implicated in the development of acute renal failure, as its free radical properties contribute to tubular cell injury [34]. Specifically, NO can exacerbate renal damage by reacting with superoxide anions to generate peroxynitrite, a highly cytotoxic species capable of inducing tubular cell destruction and subsequent renal dysfunction [35]. The observed reduction in NO levels following harmine treatment may be associated with suppression of inducible nitric oxide synthase (iNOS) expression [36]. Increased NO production is largely driven by reactive oxygen species-mediated activation of nuclear factor-kappaB (NF- κ B), which subsequently enhances iNOS transcription [8].

In the present study, co-administration of harmine with methotrexate significantly attenuated NO elevation compared with the methotrexate-only group. Similar findings have been reported by Morsy *et al.*, who demonstrated that curcumin, a potent natural antioxidant, significantly reduced lipid peroxidation and NO levels in methotrexate-intoxicated animals. Additionally, curcumin markedly enhanced renal antioxidant enzyme activities, including glutathione peroxidase and superoxide dismutase (SOD), relative to methotrexate-treated controls [18].

To further elucidate the molecular mechanisms underlying the protective effects of harmine against methotrexate-induced nephrotoxicity, the renal mRNA expression levels of heme oxygenase-1 (Ho-1), nuclear factor erythroid 2-related factor 2 (Nrf2), NAD(P)H:quinone oxidoreductase 1 (Nqo1), and thioredoxin-1 (Trx1) were evaluated using quantitative real-time PCR. As illustrated in **Figure 4**, administration of methotrexate at a dose of 20 mg/kg resulted in a significant downregulation of Ho-1, Nrf2, Nqo1, and Trx1 expression in kidney tissue ($p < 0.001$).

The immune system is capable of modulating the expression of cytoprotective enzymes in response to harmful stimuli as an adaptive defense mechanism. Nrf2 plays a pivotal role in regulating the transcription of genes involved in cellular protection against oxidative and electrophilic stress [37]. Numerous studies have highlighted the essential physiological function of Nrf2 in safeguarding renal tissue from various pathological conditions [28, 38]. Activation of Nrf2 promotes detoxification processes and elimination of reactive oxygen species, which are critical events in the prevention of renal injury [28]. Consequently, pharmacological activation of Nrf2 has been proposed as a promising therapeutic strategy for the treatment of kidney diseases. In support of this concept, a large multicenter clinical trial demonstrated that patients with type II diabetes and chronic kidney disease experienced improvement following treatment with bardoxolone methyl, a potent Nrf2 activator [39].

In the present study, methotrexate administration markedly suppressed renal Nrf2 expression in intoxicated mice ($p < 0.001$). In contrast, treatment with harmine at a dose of 20 mg/kg significantly restored Nrf2 expression levels ($p < 0.001$).

It is well established that the expression of thioredoxin reductase 1 (TXNRD1), Nqo1, and Ho-1 is regulated by Nrf2 through its binding to antioxidant response elements, initiating a cascade of cytoprotective signaling events that ultimately enhance cellular resistance to oxidative stress [40]. Mahmoud *et al.* reported that excessive ROS production induced by methotrexate led to impaired Nrf2 expression and nuclear translocation, which was accompanied by downregulation of Nrf2-dependent genes such as Ho-1 and Nqo1. Furthermore, their findings demonstrated that ferulic acid, acting as an antioxidant, activated the Nrf2/Ho-1 signaling pathway in rat kidneys, resulting in improved antioxidant defense capacity [9].

Table 2. Histopathological grading of renal alterations following methotrexate and harmine administration in mice.

Experimental group	Tubular lumen enlargement	Cytoplasmic vacuole formation	Renal vascular congestion	Tubular epithelial cell loss	Fibrotic changes in glomeruli
Control	1	1	1	1	0
Methotrexate (20 mg/kg)	3	3	4	2	3
Harmine (20 mg/kg)	1	1	1	1	0
Methotrexate (20 mg/kg) + Harmine (5 mg/kg)	3	2	4	2	2
Methotrexate (20 mg/kg) + Harmine (10 mg/kg)	2	1	2	1	1

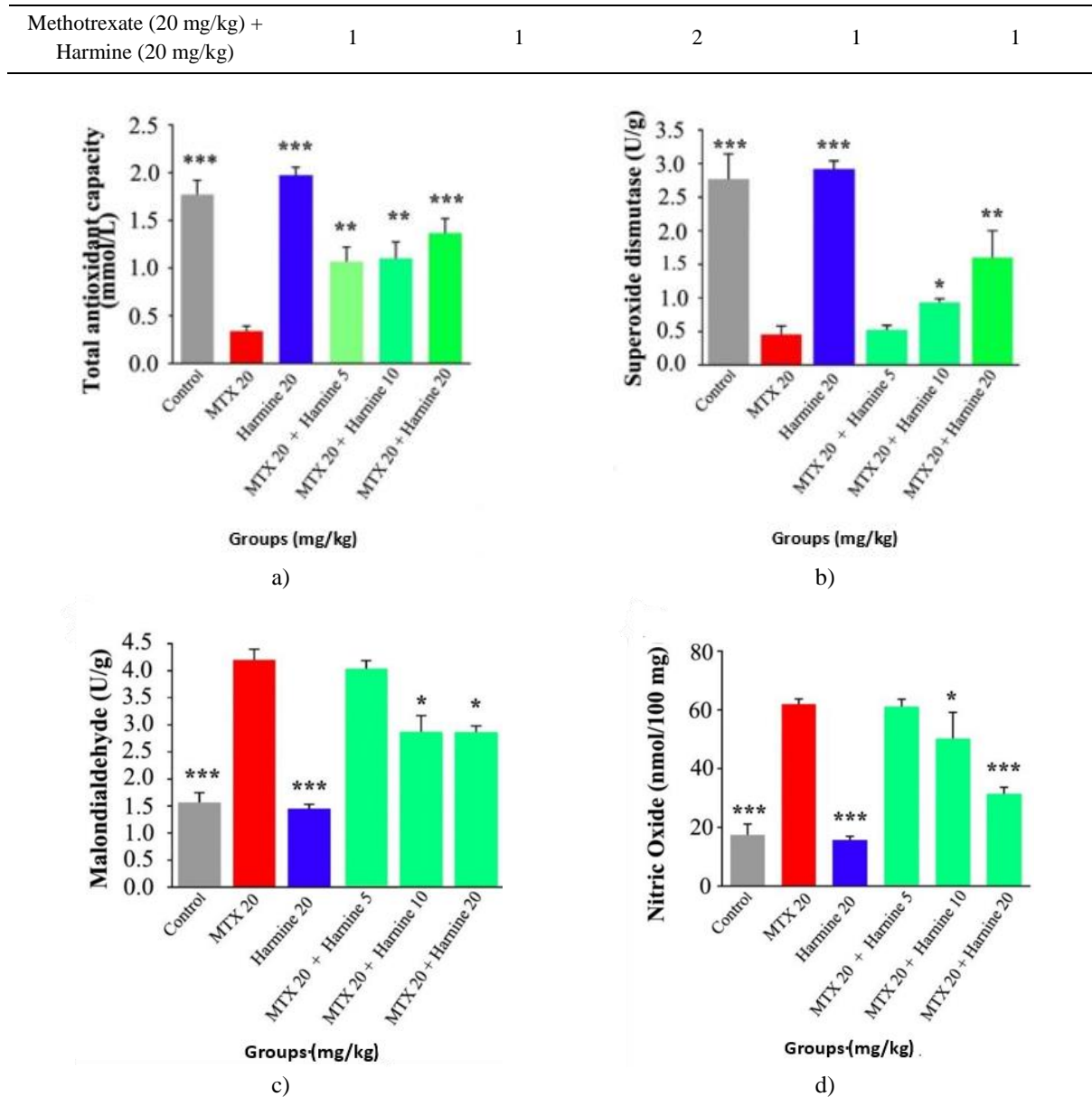


Figure 3. Influence of harmine (5, 10, and 20 mg/kg) and methotrexate (20 mg/kg) on renal oxidative stress indices in mice. Values are presented as mean \pm SEM ($n = 7$). Statistical significance was defined as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ relative to the methotrexate-treated group. MDA: malondialdehyde; TAC: total antioxidant capacity; MTX 20: methotrexate (20 mg/kg); SOD: superoxide dismutase; harmine 5, 10, 20: harmine administered at 5, 10, and 20 mg/kg, respectively.

Heme oxygenase-1 (HO-1) is widely upregulated in numerous experimental models of renal disorders and is recognized as a critical cytoprotective enzyme involved in the modulation of oxidative stress responses [40, 41]. In the present study, methotrexate exposure markedly reduced HO-1 mRNA expression in renal tissue. In contrast, administration of harmine at a dose of 20 mg/kg significantly elevated HO-1 transcript levels ($p < 0.001$). Furthermore, combined treatment with harmine and methotrexate significantly restored HO-1 expression compared with methotrexate alone ($p < 0.01$). NAD (P) H quinone oxidoreductase-1 (Nqo1) is rapidly upregulated in cells exposed to various stressors, particularly oxidative insults. Regulation of Nqo1 gene expression in both humans and mice is largely dependent on antioxidant response elements (AREs) located within its promoter region, which play a central role in maintaining redox balance and enabling cellular adaptation to oxidative stress [42]. Activation of Nqo1 expression is strongly influenced by Nrf2 signaling [43]. Our findings demonstrated a significant elevation of Nqo1 mRNA levels in the harmine-treated group ($p < 0.001$). Moreover, harmine co-treatment effectively counteracted the suppressive effect of methotrexate on Nqo1 expression ($p < 0.05$).

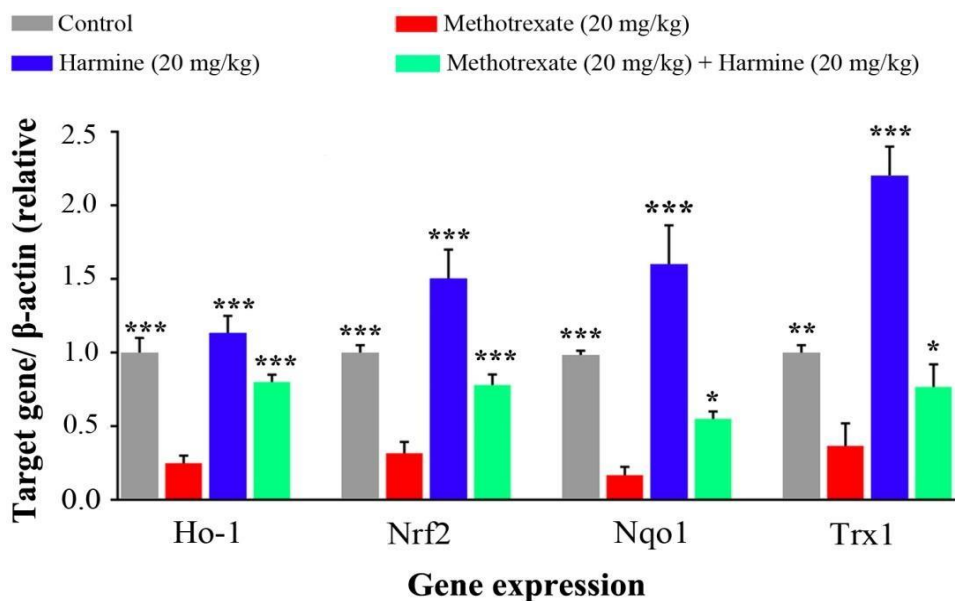


Figure 4. Impact of harmine (5, 10, and 20 mg/kg) and methotrexate (20 mg/kg) on renal expression of oxidative stress-associated genes in mice. Results are presented as mean \pm SEM ($n = 7$). Statistical significance was defined as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ in comparison with the methotrexate-treated group. Ho-1: heme oxygenase-1; NRF2: nuclear factor erythroid 2-related factor 2; Nqo1: NAD (P) H quinone dehydrogenase 1; TRX1: thioredoxin-1.

Thioredoxin-1 (Trx1) is a highly expressed endogenous antioxidant protein present in nearly all eukaryotic cells and plays a central role in maintaining redox balance as well as neutralizing reactive oxygen species (ROS) [44]. Previous investigations have shown that oxidative stress associated with ischemic injury leads to the release of Trx from renal tubular epithelial cells into the urine [45]. Regarding the interaction between Nrf2 and the thioredoxin system, activation of Nrf2-dependent antioxidant pathways enhances ROS clearance and stabilizes the thioredoxin/thioredoxin-interacting protein (TXNIP) balance, thereby limiting ROS-driven activation of NF- κ B signaling [46].

In the present study, co-administration of harmine with methotrexate resulted in elevated renal Trx1 expression, which was accompanied by reduced ROS generation, indicating an antioxidant-mediated protective effect of harmine. Methotrexate alone significantly suppressed Trx1 gene expression in kidney tissue ($p < 0.001$); however, this reduction was significantly alleviated following harmine co-treatment ($p < 0.05$). Moreover, administration of harmine alone led to a marked upregulation of Trx1 mRNA levels in renal tissue ($p < 0.001$).

It has been widely demonstrated that the restorative effects of both natural and synthetic compounds in experimental models of tissue damage are largely attributable to their antioxidant properties [47–51]. An additional potential mechanism underlying the renoprotective action of harmine may involve its influence on systemic blood pressure regulation [52]. Nevertheless, in the current investigation, no direct evidence was obtained to support a role for blood pressure modulation in harmine-mediated improvement of methotrexate-induced renal injury. Consequently, future studies are warranted to evaluate hemodynamic parameters, including blood pressure, in similar experimental models.

Conclusion

Methotrexate administration provoked pronounced oxidative stress in the kidneys of mice, whereas harmine, as a plant-derived antioxidant, effectively counteracted methotrexate-induced ROS overproduction and strengthened endogenous antioxidant defense systems. These findings suggest that harmine may serve as a promising protective candidate for preventing or reducing methotrexate-associated nephrotoxicity.

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Conflict of Interest: None

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Ethics Statement: None

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