Research Journal of Pharmacognosy (RJP) 8(4), 2021: 9–19
Received: 10 Feb 2021
Accepted: 31 July 2021
Published online: 8 Aug 2021
DOI: 10.22127/RJP.2021.272797.1676

Original article

Harmine Has Nephroprotective Effect Against Methotrexate-Induced Injury in Mice via Inhibition of Oxidative Stress

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Abstract

Background and objectives: Despite clinical use, the efficacy of methotrexate is often limited by some adverse effects, mainly nephrotoxicity. The most common mechanism of methotrexate-induced kidney damages is oxidative stress. Harmine as a plant-derived compound has antioxidant and anti-inflammatory properties, The aim of this study was to evaluate the therapeutic effect of harmine, against methotrexate -induced nephrotoxicity. **Methods:** The mice were divided into six groups: control (saline only); 20 mg/kg methotrexate; 20 mg/kg harmine, and 20 mg/kg methotrexate + harmine at three doses of 5, 10, or 20 mg/kg. Administrations were intraperitoneally and the treatment period was a 14-days. After this time, the sera and kidneys were collected from each group for the following analyses. Samples were analyzed by hematoxylin-eosin (H&E) staining, qRT-PCR, and biochemical assays. **Results:** The mice that received methotrexate showed significant increase in creatinine and blood urea nitrogen levels, and 10, or 20 mg/kg harmine mitigated these results. The number and diameter of glomeruli were improved by harmine in methotrexate -treated groups. Moreover, malondialdehyde and nitric oxide levels showed significant increase in the kidney of the mice that received methotrexate, while total antioxidant capacity and superoxide dismutase were diminished. Harmine treatment suppressed oxidative stress markers and also enhanced antioxidant defense parameters. Harmine inhibited methotrexate-induced oxidative stress as shown by the decreased expression of Nqo1, Ho-1, Trx1 and Nrf2 at mRNA level. Harmine also ameliorated histological alterations induced by methotrexate. **Conclusion:** Our results suggested that harmine has the potential to protect against methotrexate-induced nephrotoxicity.

Keywords: harmine; methotrexate; oxidative stress; toxicity

Citation: Jalili C, Darakhshan S, Akhshi N, Abdolmaleki A, Abdi A, Ghanbari A. Harmine has nephroprotective effect against methotrexate-induced injury in mice via inhibition of oxidative stress. Res J Pharmacogn. 2021; 8(4): 9–19.

Introduction

Drug-induced nephrotoxicity is a frequent side effect and also a significant cause of acute kidney injury [1]. Methotrexate is a relatively small molecule chemotherapeutic agent widely used for management of several types of cancers as well as inflammatory diseases [2-6]. The therapeutic use of methotrexate is often limited by its well-recognized adverse effects, including nephrotoxicity [3, 7-9]. Since most of methotrexate is excreted unchanged via the kidneys [10], methotrexate treatment, particularly at high doses, may cause renal failure [2]. The

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methotrexate-induced nephrotoxicity is believed to be mediated by two major mechanisms [11]. methotrexate-induced crystalline nephropathy, which occurs when methotrexate and its metabolites precipitate within the tubular lumen is characterized by obstruction of renal tubules and diminished clearance. On the other hand, this may further decrease the renal elimination of methotrexate leading to increased plasma levels and more toxicity [12]. The second mechanism of methotrexate-induced renal injury is direct tubular toxicity via oxidative stress; methotrexate causes excessive production of reactive oxygen species (ROS) in the kidney tissue [13].

In the regulation of oxidative processes, cellular systems have been equipped with a number of antioxidant defense mechanisms [14]. Several studies have revealed that methotrexate leads to a reduction in antioxidant enzymatic defense capacity in the kidney tissue. It increases lipid peroxidation, malondialdehyde (MDA) level, myeloperoxidase (MPO) activity, and nitric oxide (NO) release in the renal tissue. It also decreased catalase activity, glutathione (GSH) level, and superoxide dismutase (SOD) activity in the blood and kidney [13,15,16]. It has been reported that in addition to oxidative stress, abnormal generation of inflammatory mediators and neutrophil infiltration contribute to methotrexate-induced renal damage [16,17].

There is a great interest in expanding the clinical usefulness of methotrexate by developing new agents in order to moderate its nephrotoxicity. Today much attention has been given to the possible role of compounds of natural origin with the aim of ameliorating the side effects of methotrexate due to their antioxidant and anti-inflammatory activities. Curcumin, gallic acid, ferulic acid, caffeic acid phenethyl ester, thymoquinone, vancamine, and silymarin are some examples [9,15,17-22]. Harmine, 7-methoxy-1-methyl-9H-pyrido (3,4-b) indole, is a β-carboline alkaloid that is present in some plants such as *Peganum harmala* L. Harmine possesses several pharmacological effects, such as anti-microbial, antioxidant, and anti-tumor properties [23]. In a study, the beneficial impacts of harmine administration on kidney damage induced by nicotine were demonstrated by decreasing MDA, creatinine, and NO levels [24]. Moreover, it has been found that harmine has therapeutic effects on kidney acute injury induced by lipopolysaccharide (LPS) and protects the kidney against oxidative stress [25]. Considering the above information, in the present study, we investigated the potential protective effect of harmine against nephrotoxicity induced by methotrexate and the involvement of oxidative stress elements in this drug-induced kidney injury.

Materials and Methods

Ethical considerations

The research protocol was approved by the Ethics Committee of Kermanshah University of Medical Sciences (IR.KUMS.REC.1398.1171). All the animal experiments were carried out in accordance with the Helsinki guidelines and the Ethics Committee of Kermanshah University of Medical Sciences

Chemicals

Harmine (7-Methoxy-1-methyl-9H-pyrido[3,4-b] indole) powder was purchased from Sigma (CAS No: 442-51-3), the administrated doses were selected based on previous reports demonstrating the in vivo anti-inflammatory and antioxidant effects [25,26]. Xylazine was prepared from Alfasan co, Netherlands.The serum creatinine and blood urea nitrogen (BUN) levels were determined by kits from Pars Azmoon, Iran. Total antioxidant status was estimated by TAS, RANDOX reagents, from United Kingdom (UK). Griess Reagent System, Promega (Griess reagent) was used for nitric oxide (NO) level measurement. Trizol reagent from Invitrogen, United States was used for Total RNA extraction. Synthesized cDNA was from PrimeScript First Strand cDNA Synthesis Kit (TaKaRa Bio, Japan). Quantification of the target genes were by SYBR Green Master Mix (TaKaRa Bio Inc. Japan).

Animals and experimental protocol

Forty-two male Balb/c mice weighing 27-30 g, were exposed to 12 h light/dark cycle at normal atmospheric temperature (23 ± 2 °C) and allowed free access to food and water.

The animals were divided into six groups (7 mice in each group): control (vehicle, saline only); methotrexate (20 mg/kg) + saline; harmine (20 mg/kg); methotrexate (20 mg/kg) + harmine (5, 10, or 20 mg/kg).

The mice were treated intraperitoneally once per day for a 14-days period, with saline as the vehicle, methotrexate only, harmine only, or methotrexate + harmine. Both methotrexate and harmine were dissolved in normal saline, and

compounds were freshly prepared. Twenty-four hours after the last treatment, on day 15, all animals went under surgery. They were anesthetized with a mixture of 70 mg/kg ketamine and 10 mg/kg xylazine prior to the surgery. Blood samples were collected from the abdominal aorta into evacuated tubes and immediately centrifuged at 3,000 rpm for 15 min at 4 °C to obtain the sera. Kidney tissues were excised and separated into two parts; a part was kept for histological experiments and the other ones were immediately frozen in liquid nitrogen and stored at −80 °C for gene expression analysis.

Histopathological analysis

The kidney tissues were fixed in 10% buffered formalin for 24 h. After fixation, with a longitudinal incision, each of the kidneys was divided into two parts from the middle. Paraffinized tissue blocks were prepared for sectioning at 5-7 μm thickness and a serial section was prepared. The sections were stained with routine hematoxylin-eosin (H&E) staining and the images were captured with a light microscope (Olympus CH3, Japan). To evaluate the possible variation following treatments, the diameter of one-hundred glomeruli was measured using DP2-BSW software associated to the microscope. Also the number of glomeruli was counted in the random fields and averaged.

Biochemical assays

Creatinine and blood BUN

Serum creatinine and BUN were assessed using commercially available kits.according to the manufacturer's instructions.

Oxidative stress and antioxidant markers

Serum superoxide dismutase (SOD) and MDA levels were assessed following the methods of Nishikimi et al. and Ohkawa et al., respectively [27,28]. Total antioxidant was analyzed by Total Antioxidant Status.NO level was evaluated using Griess reagent.according to the manufacturer's instructions, and based on Giustarini et al. [29].

Quantitative transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from kidney tissue homogenate using Trizol reagent and the cDNA was synthesized by PrimeScript First Strand cDNA Synthesis Kit. The target genes were quantified by SYBR Green Master Mix and Corbett Rotor-Gene 6000 thermocycler. The results were presented as the fold change in the target gene expression normalized to β-actin as the reference gene and relative to the control group. The primer sequences are shown in Table 1.

Table 1. Primers used for qRT-PCR analysis

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

Statistical analysis

Statistical analysis was performed by SPSS Version 19.0 (SPSS, Chicago, IL, USA). The comparisons were performed using one-way ANOVA followed by Tukey's post-hoc analysis and expressed as mean ± standard error of the mean (SEM). In all cases, $p<0.05$ was considered statistically significant.

Results and Discussion

The results showed that Harmine treatment protected the mice against methotrexate-induced kidney damage by improving antioxidant defense status and up-regulating genes involved in response to oxidative stress. Methotrexate as a chemotherapeutic drug causes renal intoxication that has challenged its clinical use [11]. Studies have demonstrated the protective or ameliorative roles of phytochemicals with inherent antioxidant properties in methotrexate-induced kidney toxicity that are capable of boosting cellular anti-oxidative indices [9,17,19,20,22]. Harmine employed in the present study is a potent natural antioxidant with numerous biological activities including nephroprotective effects [25, 26]. Harmine has been reported to attenuate dioxin-induced hepatotoxicity [30], also LPS-induced acute kidney injury [25]. However, nothing has yet been performed on its potential protective/therapeutic effects to mitigate methotrexate-induced nephrotoxicity. Herein, we investigated the role of harmine in alleviating methotrexate-induced kidney injury in mice.

Studies have shown that methotrexate induced kidney injury evidenced by changes in the serum creatinine and BUN, uremia, and hematuria [9,31].

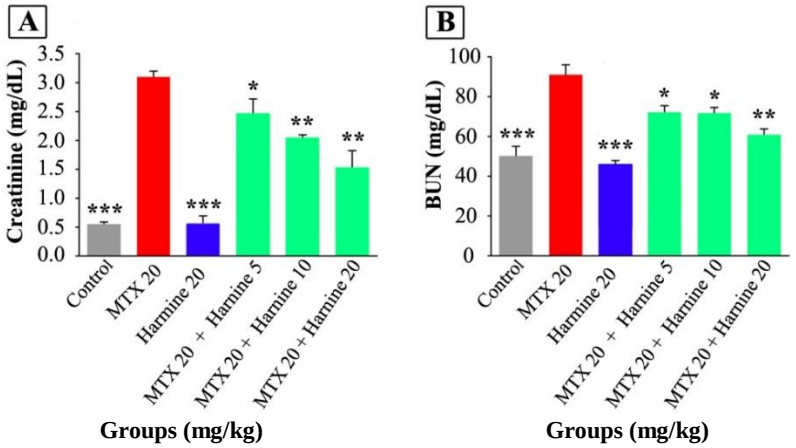

Figure 1. Effects of harmine (5, 10, 20 mg/kg) and methotrexate (20 mg/kg) on the parameters related to the function of kidney. Data are expressed as mean ± SEM; n = 7; *p<0.05; **p<0.01; ***p<0.001 compared to methotrexate group. BUN: blood urea nitrogen; MTX 20: methotrexate (20 mg/kg); harmine 5, 10, 20: harmine at doses of 5, 10, 20 mg/kg, respectively.

We demonstrated that methotrexate induced a significant increase (p<0.001) in serum creatinine (Figure 1A) and BUN (Figure 1B) levels compared to the control group.

The mice that received 10 or 20 mg/kg harmine showed significantly decreased levels of creatinine and BUN when compared with methotrexate-untreated group (p<0.05 for 10 mg/kg and p<0.01 for 20 mg/kg). The ameliorative effect of harmine on methotrexate-induced nephrotoxicity was further confirmed by the histological evaluation. Assessing the control group revealed the normal structure of the glomeruli and renal tubules (Figure 2A). However, the administration of methotrexate caused tubular cell detachment, dilation of renal tubules, vascular congestion, glomerular fibrosis, and intra-cellular vacuolization (Figure 2B). The harmine-treated mice (at a dose of 20 mg/kg) were in a similar situation to the control group (Figure 2C). Harmine (20 mg/kg) protected kidney tissue against mentioned damages induced by methotrexate, as depicted in Figure 2D. The histological indices are shown in Table 2. We also evaluated the glomeruli by quantifying the number and diameter of these damages. methotrexate administration also caused a significant reduction in the number of glomeruli (p<0.001), and the diameter of glomerulus (p<0.01). The results showed that harmine treatment improved the number and the diameter of glomeruli in methotrexate-treated mice (Figures 2E, F).

Based on the existing documentation, the most common mechanism involved in the pathogenesis of methotrexate nephrotoxicity is oxidative stress which is accompanied by inflammation [8,9,13,22]. Excess ROS production, diminished cellular antioxidants, and lipid peroxidation are hallmarks of methotrexate-induced injury in liver, brain, and kidney [8,9]. This ROS production triggered by methotrexate could be explained by increased infiltration and activation of neutrophils [32], mitochondrial dysfunction [33], and upregulation of NADPH oxidase [34]. In addition, ROS can induce the release of pro-inflammatory cytokines by activating the nuclear factor-kappaB (NF-κB), tumor necrosis factor alpha (TNFα), interleukins (IL)-1β, and -10 [21,35]. These modulatory factors display important roles in methotrexate nephrotoxicity [21]. Suppressing ROS generation and enhancement of cellular antioxidant defenses can mitigate methotrexate-induced kidney injury [19,21], and harmine was considered as an antioxidant agent in this study. Total antioxidant capacity (TAC), also, MDA and SOD levels were assessed to evaluate the oxidative stress induced by methotrexate and the protective effect of harmine (Figure 3). The value of TAC was markedly decreased in the mice that received methotrexate compared to the control group (p<0.001). The groups that received 5, 10, or 20 mg/kg harmine showed significant improvement in TAC levels (Figure 3A). In methotrexate group, the oxidative stress marker, MDA, significantly increased compared to the control (p<0.001); whereas treatment with either 10, or

20 mg/kg of harmine could significantly protect the kidney against lipid peroxidation ($p<0.05$; Figure 3B). In a study conducted by Salahshoor et al., harmine administration mitigated MDA, BUN, creatinine, and NO levels towards the normal control group in nicotine-induced kidney dysfunction [24]. Following 14 days of methotrexate administration, the level of SOD antioxidant marker significantly decreased compared to the control group ($p<0.001$). In Methotrexate plus 20 mg/kg harmine group, harmine treatment reversed SOD significantly compared to the control group ($p<0.01$; Figure 3C). In agreement with this finding, a study done by Hassanein et al. reported that methotrexate resulted in an increase in the MDA contents in the rat model of lung injury [22]. Also, Niu et al. reported that Methotrexate resulted in a decrease in GSH content and enzymatic activity of SOD in hepatic, renal, and cardiac injuries. However, pre-treatment with 25 or 50 mg/kg harmine markedly alleviated kidney injury by reducing the formation of MDA and MPO while increasing SOD and GSH in acute kidney injury induced by LPS in mice [25].

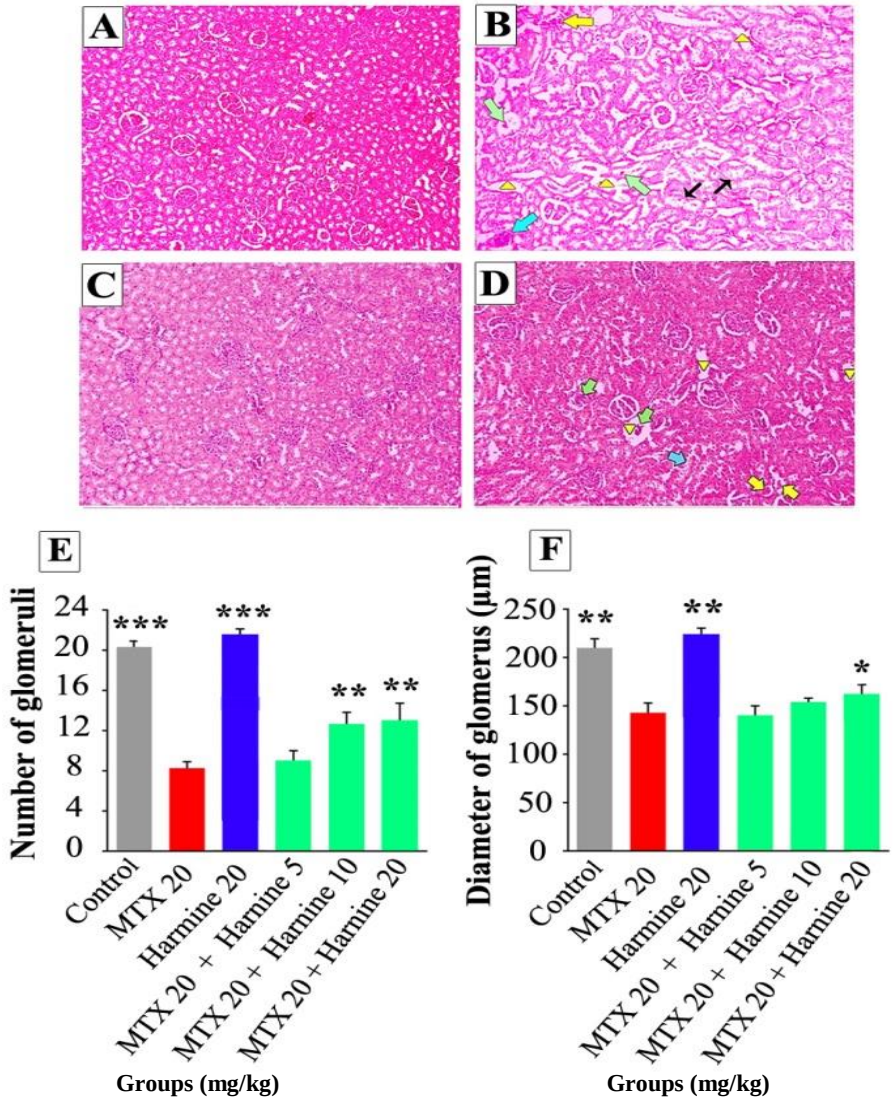

Figure 2. Effects of harmine (5, 10, 20 mg/kg) and methotrexate (20 mg/kg) on the histopathological changes in the kidney of mice. A-D: H&E staining of the kidneys; A: control group; B: methotrexate-treated group (yellow, green, black and blue arrow showing proteanous cast, tubular cell detachments, intra-cellular vacuolization, and glomerular fibrosis, respectively. Yellow triangle demonstrates the dilation of renal tubules), C: harmine (20 mg/kg) treated group, D: methotrexate (20 mg/kg) + harmine (20 mg/kg) group, E: quantification the number of glomeruli; F:) diameter (µm) of glomeruli; *p<0.05; **p<0.01; ***p<0.001 compared to Methotrexate group. MTX 20: methotrexate (20 mg/kg); harmine 5, 10, 20: harmine at doses of 5, 10, 20 mg/kg, respectively.

Mice that received Methotrexate showed a significant increase in NO levels ($p<0.001$), whereas NO level lowered by harmine doses but was significant at 20 mg/kg ($p<0.001$; Figure 3D). NO has a role in acute renal failure because of the fact that the free radical nature of NO might participate in the tubular damage [34]. NO increases renal injury through its reaction with superoxide radical and generation of a cytotoxic peroxynitrite [35], which can damage the tubular cells causing renal failure. The decrease in NO level may be due to a reduction in inducible nitric oxide synthase (iNOS) level [36]. ROS-mediated activation of NF-κB and subsequent up-regulation of iNOS are the main mechanisms behind increased NO levels [8].

Harmine, when co-administrated with methotrexate, showed a declined level of NO compared to the methotrexate-induced injury group. In a study by Morsy et al., curcumin as a natural potent antioxidant significantly suppressed both lipid peroxidation and the elevation of NO levels in comparison with methotrexate-intoxicated group. Moreover, curcumin treatment significantly increased renal glutathione peroxidase and SOD activities compared to methotrexate-treated group [18].

For further clarification of the mechanisms underlying ameliorative effects of harmine on methotrexate nephrotoxicity, mRNA expression of heme oxygenase-1 (Ho-1), nuclear factor erythroid 2-related factor 2 (Nrf2), quinone oxidoreductase (Nqo1), and Trx1 was determined in renal tissue by qRT-PCR. As presented in Figure 4, the administration of methotrexate at 20 mg/kg resulted in a significant down-regulation of Ho-1, Nrf2, Nqo1, and Trx1 genes in the kidney of the mice ($p<0.001$). The immune system is able to alter the levels of cellular protective enzymes in response to specific stimuli as a defense mechanism. Nrf2is an important factor that adjusts the production of proteins related to protection in response to harmful external stresses [37]. In studies, a vital physiological role for Nrf2 is considered in the protection of the kidney against a number of problems [28,38]. The cytoprotective effects of Nrf2 activation involve the elimination of toxins and ROS which have considerable importance in renal toxicity [28]. It is believed that Nrf2 enhancers and/or activators can be promising innovative classes of candidate therapeutic agents for treatment of renal problems. The results of a large multi-center trial revealed that patients with type II diabetes and chronic kidney disease recovered with a potent Nrf2 activator, bardoxolone methyl [39]. We found that methotrexate significantly reduced kidney Nrf2 in methotrexate-intoxicated mice ($p<0.001$), while treatment with 20 mg/kg harmine caused a significant increase in its expression ($p<0.001$).

It has been established that the expression of thioredoxin reductase 1 (TXNRD1), NAD(P)H: (Nqo1, and Ho-1 are regulated through Nrf2 binding. This activates a cascade of events which, finally, affect the oxidative status of the cells and provide robust protection against oxidative challenge [40]. In a study conducted by Mahmoud et al., it was revealed that the diminished Nrf2 expression and translocation, as a consequence of methotrexate-induced excessive generation of ROS, were associated with down-regulation of HO-1 andNqo1, genes directly regulated through Nrf2. They also showed that ferulic acid as an antioxidant activated the Nrf2/HO-1 pathway in the kidney of rats which was positively correlated with enhanced antioxidant defenses [9].

Table 2. The grading of histopatological changes in kidney after administration of methotrexate and harmine in mice

	Tubular dilatation	Intra-cellular vacuolization	Vascular congestion	Tubular cell detachment	Glomerular fibrosis
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
Methotrexate (20 mg/kg) + harmine (20 mg/kg)	1	1	2	1	1

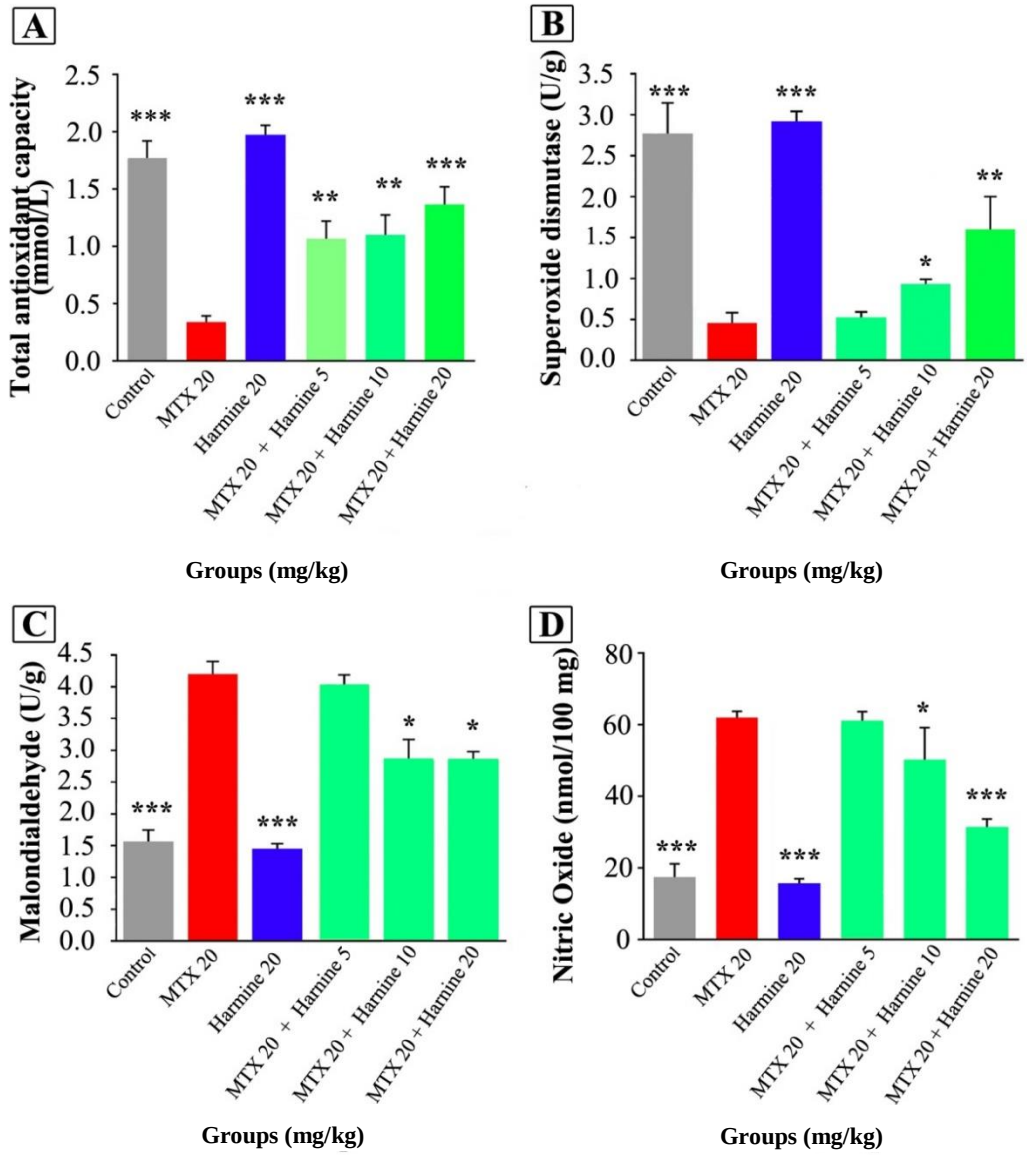

Figure 3. Effects of harmine (5, 10, 20 mg/kg) and methotrexate (20 mg/kg) on the oxidative stress related parameters of mice kidney. Data are expressed as mean ± SEM; n = 7; *p<0.05; **p<0.01, ***p<0.001 compared to methotrexate group. TAC: total antioxidant capacity; MDA: malondialdehyde; SOD: superoxide dismutase; MTX 20: methotrexate (20 mg/kg); harmine 5, 10, 20: harmine at doses of 5, 10, 20, mg/kg respectively.

HO-1 is induced in a variety of kidney disease models and plays an important protective role in regulating oxidative stress [40,41]. Our results showed a decrease in mRNA abundance of HO-1 in the mouse kidney as a result of methotrexate administration. Nonetheless, treatment of mice with 20 mg/kg of harmine significantly increased mRNA abundance of Ho-1 ($p<0.001$). Co-administration of harmine with methotrexate produced a significant amelioration of Ho-1 expression level compared to methotrexate group ($p<0.01$). Nqo1cellular level is increased quickly under a variety of stress responses including oxidative stress. The expression of Nqo1 gene in human and mouse are primarily regulated via ARE sequences in the promoter region, which controls redox homeostasis and facilitates adaptation of most cells to oxidative stress [42]. Induction of Nqo1to high levels in cells can be mediated by Nrf2 [43]. The results revealed significant improvement in Nqo1 expression in harmine group ($p<0.001$); also, decreased effect of methotrexate on Nqo1 was mitigated by harmine co-administration ($p<0.05$).

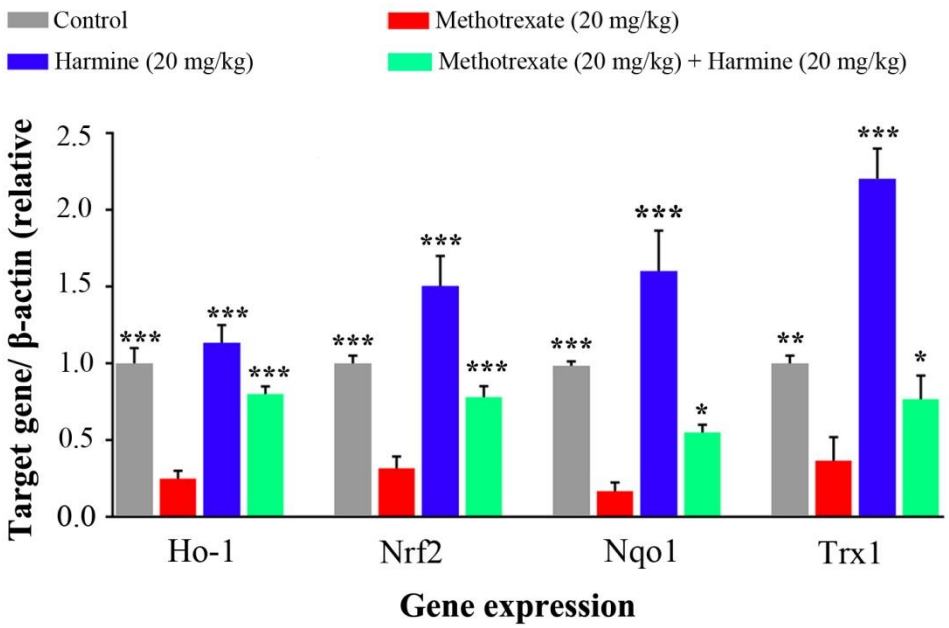

Figure 4. Effects of harmine (5, 10, 20 mg/kg) and methotrexate (20 mg/kg) on the expression of genes involved in oxidative stress in the mice kidney. Data are expressed as mean ± SEM; n = 7; *p<0.05; **p<0.01, ***p<0.001 compared to methotrexate group. Ho-1: heme oxygenase-1; NRF2: nuclear factor erythroid 2-related factor 2; Nqo1: NAD(P)H quinone dehydrogenase 1; TRX1: Thioredoxin 1

Thioredoxin (Trx)1 is an abundant antioxidant protein expressed in almost all eukaryotic cells and plays a vital role in redox signaling and serves as a ROS scavenger [44]. It has been reported that Trx is secreted from renal tubular cells into urine due to oxidative stress caused by ischemia injury [45]. In explaining the relationship between Nrf2 and Trx, it can be assumed that an enhanced antioxidant response mediated via Nrf2 activation scavenges ROS and maintains thioredoxin interacting protein (TXNIP)/thioredoxin, resulting in suppression of ROS-priming NF-κB signaling [46]. Herein, an increase in Trx1 in the kidney of mice co-treated with methotrexate and harmine was accompanied by suppressed ROS production via harmine effect. Decreased expression of Trx1 in the kidney of mice that received methotrexate ($p<0.001$) was moderated by harmine administration in co-treatment group ($p<0.05$). In addition, harmine-only injection caused a significant increase in Trx1 mRNA expression in the kidney tissue ($p<0.001$). The recovery effect of neutral and chemical compounds on tissue injury models has been shown mainly by antioxidant activity [47-51]. Another possible protection of harmine should be modulation of blood pressure [52]. Although harmine has a modulatory effect on blood pressure, we found no evidence that shows the impact of this property of Harmine for improvement in the kidney injury models. Therefore, it is recommended to check blood pressure in these models as well.

Conclusion

Methotrexate stimulated oxidative stress in the kidney of mice and harmine as a natural antioxidant markedly prevented methotrexate-induced overproduction of ROS, and enhanced antioxidant defenses in kidney injury. Therefore, harmine may represent a promising protective agent against methotrexate-induced nephrotoxicity.

Acknowledgments

This study was funded by Kermanshah University of Medical Sciences (Grant number 981023).

Author contributions

Cyrus Jalili was involved in conceptualization, data curation, and validation and funding acquisition; Sara Darakhshan performed the analysis and prepared the original draft of the

manuscript, Nasim Akhshi contributed to investigations, writing and editing of the manuscript; Amir Abdolmaleki contributed to final editing and responding to reviewers. Abdolnasir Abdi performed the experiments; Ali Ghanbari contributed in project administration, supervision, writing and editing of the manuscript, validation and funding acquisition. All authors read, critically reviewed, and approved the final manuscript.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

MDA: malondialdehyde; MPO: myeloperoxidase; GSH: glutathione; BUN: blood urea nitrogen; SOD: superoxide dismutase; H&E: hematoxylin-eosin; BUN: urea nitrogen; NO: nitric oxide; ROS: reactive oxygen species; qRT-PCR: quantitative transcription polymerase chain reaction; TNFα: tumor necrosis factor alpha; TAC: total antioxidant capacity, TXNRD1: thioredoxin reductase 1, Ho-1: heme oxygenase-1; Nqo1: NAD(P)H quinone dehydrogenase 1; Nrf2: nuclear factor erythroid 2-related factor; Trx: thioredoxin