Harmine Mitigates Liver Injury Induced by Mercuric Chloride via the Inhibition of Oxidative Stress

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Abstract
Background and objective: The mercury-induced liver pathogenesis is mainly mediated by oxidative stress. The aim of the current study was to evaluate the possible ameliorative effect of harmine, a natural compound, on liver toxicity induced by mercuric chloride (HgCl₂).

Methods: Forty-two male Balb/c mice were randomly divided into six groups (n = 7): Control, HgCl₂ (0.5 mg/kg), harmine (20 mg/kg), and HgCl₂ (0.5 mg/kg) + harmine (5, 10, or 20 mg/kg). The mice received treatments once per day for two weeks. After this period, the blood and tissue samples were collected for analyses.

Results: HgCl₂ caused a significant increase in levels of hepatic enzymes alanine aminotransferase, aspartate transaminase, and alkaline phosphatase; while harmine ameliorated these effects. Harmine in HgCl₂-intoxicated mice, showed protective effects as evidenced by the increase in liver relative weight to body as well as the diameter of central vein in the co-treated group. Serum levels of malondialdehyde and nitric oxide increased in HgCl₂, while they were declined in harmine co-treated groups compared to HgCl₂ group. The serum level of superoxide dismutase and total antioxidant capacity improved following harmine treatment in the co-administered group compared to HgCl₂ group. Moreover, gene expression analysis demonstrated that harmine treatment improved the HgCl₂-induced decreasing of Ho-1, Nrf2, Hqo1, and Trx1. The histopathological examination confirmed the protective effects of harmine.

Conclusion: Mercury can induce toxicity by elevation of oxidative stress in the liver and harmine attenuates hepatic injury induced by HgCl₂, at least in part, through its antioxidant activities.

Keywords: harmine; hepatotoxicity; liver; mercuric chloride; oxidative stress


Introduction
Mercury (Hg), a heavy metal, is a ubiquitous pollutant with toxic effects in any form of life. It is derived from natural sources and human activities and is easily distributed in air, water, and soil [1]. Since mercury is abundant in the environment, it is nearly impossible for most people to avoid its exposure; hence, poisoning from occupational exposure and environmental pollution continues to be a concern [2]. High levels of Hg exposure cause toxic effects through progressive irreversible accumulation in tissues and organs, including the nerves, cardiovascular, reproductive and hepatic tissues, and the immune system [3,4]. The liver is the major site for metabolism and detoxification, and previous studies have shown that the liver is the mostly affected organ by mercury exposure [5]. A central mechanism of hepatic toxicity caused *Corresponding author: aghanbari@kums.ac.ir
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by mercuric chloride (HgCl\(_2\)) is associated with the oxidative stress [5-8]. Previous studies have shown that HgCl\(_2\) inhibits the activities of free radical scavenging systems in the liver. It is found that HgCl\(_2\) elevates biochemical parameters of the liver such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), gamma glutamyl transferase (GGT), and bilirubin, while the level of total protein is significantly declined [7]. It has also suppressed the antioxidant defense system such as activities of catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) and has increased the hepatic malondialdehyde (MDA) concentration [9].

In recent years, the use of natural antioxidants in the treatment of heavy metal poisoning has become widespread [10]. Harmin as one of the active plant-derived compounds, is a tricyclic alkaloid. It possesses a wide range of ameliorative/protective properties, and studies confirmed that harmine has antioxidant and anti-inflammatory activities in some organs [11-13]. A study displayed harmine could mitigate lipopolysaccharide (LPS)-induced acute kidney injury through inhibition of oxidative stress and inflammation [12]. In another study, harmine showed beneficial effects on nicotine-induced liver failure in mice the prevention of oxidative stress [13]. Considering harmine role in oxidative stress and inflammatory states, it is assumed that this compound can ameliorate the oxidative stress caused by HgCl\(_2\) in hepatic tissue. Hence, the aim of this work was to explore the potential protective effects of harmine on HgCl\(_2\)-induced liver injury and its underlying mechanisms in regulating oxidative stress.

Materials and Methods

Ethical considerations

The study protocol was approved by the Ethics Committee of Kermanshah University of Medical Sciences (IR.KUMS.REC.1398.945; June 2019) and the animal care was in accordance with the related guidelines.

Chemicals

Harmine (7-Methoxy-1-methyl-9H-pyrido[3,4-b] indole) powder was purchased from Sigma, USA (CAS No: 442-51-3). The levels of MDA and SOD were determined by commercial kits from ZellBio (GmbH, Germany). Total antioxidant capacity (TAC) was measured by Total Antioxidant Status kit (TAS, RANDOX Reagents, UK). The ALT, ALP, and AST serum levels were detected using related Pars Azmoon kits levels were measured according to the manufacturer’s instructions. Nitric oxide (NO) level was evaluated using Griess reagent (Griess Reagent System, Promega; USA).

Animals and experimental protocol

In this study, forty-two male Balb/c mice (27-30 g) were housed in laboratory conditions (12 h of lightness/12 h of darkness, 21-23 °C) with ad libitum access to water and food. The animals were randomly divided into six groups (n = 7): Control (vehicle, saline only); HgCl\(_2\) (0.5 mg/kg) + vehicle; vehicle + harmine (20 mg/kg); and HgCl\(_2\) (0.5 mg/kg) + harmine (5, 10, or 20 mg/kg).

For randomization, we applied the completely randomized (between subjects) design. In this design, one of the groups is considered as control, and the animals are assigned to the treatment groups strictly at random. Besides, the positions within the animal house and the order in which the treatments are given and the outcome is measured should also be in random order. Harmine was administrated one hour later than HgCl\(_2\) treatment. The doses of HgCl\(_2\) and harmine were selected based on previous reports demonstrating the in vivo anti-inflammatory and antioxidant effects [14,15]. The mice received intraperitoneal saline, HgCl\(_2\), and harmine once per day for two weeks. Twenty-four hours after the last treatment, all animals were anesthetized with a mixture of 70 mg/kg ketamine and 10 mg/kg xylazine (Alfasan, Holland). Blood samples were collected from the abdominal aorta into evacuated tubes and were immediately centrifuged at 3,000 rpm for 15 min at 4 °C to obtain the sera. For histological analysis, a portion of tissues was immersed in 10% buffered formalin, and a portion of samples was immediately frozen in liquid nitrogen and stored at −80 °C until use.

Biochemical analysis

Serum samples were assessed for the parameters related to oxidative stress including MDA, SOD, and total antioxidant capacity (Vagvala & O’Connor). The levels of MDA and SOD were determined by commercial kits (ZellBio GmbH, Germany) according to the manufacturer’s instructions by a microplate reader (STAT FAX
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2100, USA). TAC was measured by Total Antioxidant Status kit (TAS, RANDOX Reagents, UK) using a UV/VIS Spectrophotometer (UNICO SQ2800, USA). Alanine aminotransferase (ALT), alkaline phosphatase (ALP), and aspartate aminotransferase (AST) serum levels were detected using related Pars Azmoon kits according to the manufacturer’s instructions. Nitric oxide (NO) level was evaluated using Griess reagent (Griess Reagent System, Promega) according to the manufacturer’s instructions.

Histological examination
The liver tissues were fixed in buffered formalin 10% at 4 °C, embedded in paraffin, sectioned at 6-8 µm, and then processed for hematoxylin and eosin (H&E) staining. After staining, images were captured using a light microscope (Olympus CH3, Japan). To evaluate the possible variation following treatments, the diameter of one-hundred central vein was measured using DP2-BSW software associated to the microscope in random fields and averaged.

RNA isolation and qRT-PCR
Liver tissues were taken from the animals and stored in liquid nitrogen until processed for RNA isolation. Total RNA was extracted from all groups using a standard RNA extraction protocol (TRIzol reagent, Invitrogen). RNA was reverse transcribed using PrimeScript First Strand cDNA Synthesis Kit (TaKaRa Bio). Quantitative RT-PCR (qRT-PCR) was performed using SYBR Green Master Mix (TaKaRa Bio Inc.) and Corbett Rotor-Gene 6000 thermocycler. Expression levels of target genes were normalized against beta-actin mRNA level, and the control group was expressed as 1 to indicate a precise fold change. Features of the primers are shown in Table 1.

Statistical analysis
Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA; version 19.0). The comparisons were performed using one-way ANOVA followed by Tukey’s post-hoc and expressed as mean ± standard error of mean (SEM). A value of p<0.05 was considered as significant.

Results and Discussion
The notable role of oxidative stress in injury associated with mercuric chloride poisoning suggests that antioxidants may be beneficial to mitigate related toxicity [10,16-18]. We investigated the effect of harmine as a natural antioxidant on the HgCl₂-induced liver injury and the underlying elements involved in regulating oxidative stress. Studies established that mercury toxicity promotes the generation of reactive oxygen species (ROS) such as superoxide and hydrogen peroxides, which induce oxidative stress, resulting in lipid peroxidation and cell membrane damage of hepatocytes and activation of liver enzymes [15,19]. It is associated with binding to thiol groups in several proteins and peptides, a decrease in cellular antioxidants such as SOD, glutathione (GSH), and CAT, along with an increase in MDA [1,7,9,14,16,17,20]. In accordance with these results, as shown in Figure 1A, compared to the control group, serum MDA content was significantly increased after a 14-days period of HgCl₂ treatment (p<0.001), while adding harmine at 20 mg/kg as co-administration caused a reduction in its level (p<0.01). In the HgCl₂ group, SOD level sharply decreased (p<0.001); however, co-treatment with harmine resulted in an amelioration in generated toxicity (Figure 1B), which was significant at 20 mg/kg dose (p<0.001).

In this study, we also detected a significant decline in total antioxidant capacity in mice exposed to HgCl₂ (p<0.001).

Table 1. Primers used for qRT-PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD(P)H:quinone oxidoreductases1 (Nqo1)</td>
<td>F: AAGGATGGAAAGAAACGCTGGAGA</td>
</tr>
<tr>
<td></td>
<td>R: GC CCC ACAGA AAAGGC CAAA TT TCT</td>
</tr>
<tr>
<td>Thioredoxin 1 (Trx1)</td>
<td>F: CC TT CC TG CA CT CC TCA T CC T C</td>
</tr>
<tr>
<td></td>
<td>R: TCC CAT TCC CCT TCA AGG AAAC</td>
</tr>
<tr>
<td>Heme oxygenase-1 (Ho-1)</td>
<td>F: CCT TCC GAC AT C TG CCC GCC</td>
</tr>
<tr>
<td></td>
<td>R: GC AG CTC TCA AAA ACG T C A A C</td>
</tr>
<tr>
<td>Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)</td>
<td>F: CAG C AT GTG AGC C T T G A A</td>
</tr>
<tr>
<td></td>
<td>R: TG AG AC ACT G C T C A C T C</td>
</tr>
<tr>
<td>Actb</td>
<td>F: CAC T T CT ACA AT G G C T C G G</td>
</tr>
<tr>
<td></td>
<td>R: CT G G AT G G C T AC GTAC AT G G</td>
</tr>
</tbody>
</table>
Figure 1. Effects of harmine on oxidative/nitrative stress in the HgCl$_2$-induced liver injury. Mice that received HgCl$_2$(0.5 mg/kg) showed a significant increase in MDA (A) and NO (D) levels, also decrease in TAC (C) and SOD level (B), whereas treatment with harmine ameliorated these alterations. Data are expressed as mean ± SEM, n = 7. *p < 0.05, **p < 0.01, ***p < 0.001. TAC, total antioxidant capacity; MDA, malondialdehyde; SOD, superoxide dismutase; NO, nitric oxide.

On the other hand, at all three doses used in this experiment, harmine showed an improvement in TAC index (Figure 1C). In LPS-induced kidney injury, pre-treatment with 25 or 50 mg/kg harmine markedly reduced the formation of MDA and myeloperoxidase (MPO), while it increased SOD and GSH activities [14]. Administration of HgCl$_2$ in mice resulted in a significant elevation in the serum levels of AST, ALT, and ALP as compared to the control group (Figure 2). Harmine at 20 mg/kg dose caused a significantly (p<0.01) decline in the level of AST in the HgCl$_2$ plus harmine treatment group. Also, harmine reduced the decreasing effect of HgCl$_2$ on ALT, significantly for both 10 and 20 mg/kg (p<0.05). Harmine also showed an ameliorative effect against nicotine-induced damage to the liver. Following nicotine administration, a major toxic component of cigarette smoke, AST, ALT, and ALP levels was elevated after a 28 days-period treatment. Also, harmine could improve thiobarbituric acid reactive species (TBARS), MDA, and NO levels in co-treated group. It can be concluded that harmine counteracted the oxidative stress of nicotine [21].
Elevations in ALT and AST is an important marker for hepatocellular damage, whereas, an elevation in ALP and bilirubin in disproportion to ALT and AST would denote a cholestatic pattern [22]. Animals in our study showed significant rises in AST and ALT enzymes in serum after HgCl$_2$ exposure. This could be due to the release of these enzymes from the cytoplasm, into the blood circulation after breaking the plasma membrane and hepatocyte injury [23]. Results obtained from a study by Joshi et al. showed that N-acetyl cysteine and selenium individually or in combination provided protection against mercury toxicity by decreasing AST, ALT, and LDH activities [23]. Alkaline phosphate (ALP) is a membrane protein and its alterations are likely to affect the membrane permeability and cause the imbalance in transport of metabolites. Moreover, Plaa et al. [24] have reported that this phosphate enzyme acts as an indicator of cholestatic changes. In our study, HgCl$_2$ administration to mice led to a marked elevation in the level of serum ALP (Figure 2) significantly for both 10 and 20 mg/kg (p<0.05), which is indicative of hepatocellular damage. In a study by Mumtaz et al., the effects of HgCl$_2$ administration and protective mechanism of ascorbic acid in heavy metals-induced hepatotoxicity was investigated in intoxicated rabbits. Supplementation of vitamin C as an antioxidant showed an ameliorating potential after 14 and 28 days by changing the alternations of ALAT, ASAT, LDH, GGT, and bilirubin parameters towards normal levels [7].

In addition, increased levels of inflammatory cytokines and NO and also impairment in the function of mitochondria are thought to complicate the harmful effects of mercury [25,26]. Biological defense produces both superoxide anion and NO during the oxidative stress triggered during inflammatory processes. Under these conditions, NO and the superoxide anion may react together to generate significant amounts of a much more active oxidative molecule, peroxynitrite anion (ONOO$^-$) [27]. In our study, compared to the control group, nitrosative stress was markedly increased in the HgCl$_2$-exposed mice as exhibited by a significant increase (p<0.001) in serum NO level. Its level, however, significantly decreased when 20 mg/kg harmine was added (p<0.01; Figure 1D). The increased serum NO level reflects inflammatory responses, whereas harmine showed anti-inflammatory effects by decreasing the secretion of this marker.

Our stereological and histopathological findings were consistent with each other. The weight of the liver decreased significantly in the HgCl$_2$-treated group compared to the liver of controls. Harmine at 20 mg/kg significantly improved the
weight of the liver compared to the related weight of HgCl₂-treated in co-administration group (Figure 3A; p<0.05). The diameter of the central vein increased significantly in the group exposed to Hg compared to controls (p<0.01), and harmine at 20 mg/kg could ameliorate this effect of HgCl₂ (Figure 3B; p<0.05). The liver of the control group appeared normal (Figure 3C). We observed enlarged blood vessels and dilated sinusoids with increased perivascular connective tissue in the HgCl₂-treated group. The volume of the parenchyma decreased, but the volume of the sinusoids increased in the HgCl₂-treated group compared to controls (Figure 3D). On the other hand, the density and total number of hepatocytes reduced in the group exposed to HgCl₂, which was resulted from the degeneration and death of these cells. The harmine-treated group (at 20 mg/kg dose) was in a similar situation to the control group (Figure 3E). However, in co-administration group, the signs of improvements towards the control group were shown. The density and total number of hepatocytes improved in the harmine co-administrated group (Figure 3F).

Figure 3. Effects of harmine on histopathological changes in the HgCl₂-induced hepatic damage. A) Liver relative weight to the body weight, and B) diameter (µm) of the central vein. H&E staining of the liver of C) control group showing the normal structure of tissue including parenchymal and non-parenchymal cells; D) HgCl₂-administrated mice showing enlarged blood vessels and dilated sinusoids with increased perivascular connective tissue. E) The harmine-treated group (at 20 mg/kg dose) was in a similar situation to the control group. F) Administration of harmine at 20 mg/kg dose protected hepatic tissue against histopathological alterations induced by HgCl₂.
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Figure 4. Effects of harmine on the expression of genes involved in oxidative stress in the liver of HgCl₂-induced injury. Administration of HgCl₂ significantly decreased mRNA level of HO-1, Nrf2, Nqo1, and Trx1. Data are expressed as mean ± SEM, n = 7. *p < 0.05, **p < 0.01, and ***p < 0.001. HO-1, heme oxygenase-1; Nrf2, nuclear factor erythroid 2-related factor 2; NQO1, NAD(P)H quinone dehydrogenase 1; TRX1, thioredoxin 1. The expression of transcripts was normalized against beta-actin, where the control group was expressed as 1 to indicate a precise fold change.

The nuclear height of the hepatocytes significantly decreased in the Hg exposed group and the nuclear diameter of hepatocytes significantly decreased. We believe that the decrease might be due to alteration of the genetic material including pyknotic or heterochromatic changes [28].

ROS levels have been shown to influence the expression of key genes involved in regulating cellular and systemic oxidative stress [29]. The effects of HgCl₂ and harmine on the mRNA level of thioredoxin (Trx), nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), and (NAD(P)H quinone dehydrogenase 1 (Nqo1) evaluated by qRT-PCR (Figure 4). The expression of all genes significantly declined following HgCl₂ exposure in the liver of exposed mice (p<0.05). Thioredoxin system consists of thioredoxin (Trx) and thioredoxin reductase (TrxR) senses and responds to oxidative stress and modulates the redox status by scavenging ROS and by regulating several signaling proteins [30]. Trx1 functions as a molecular switch turning the cellular redox state into kinase signaling; thus, it is able to regulate DNA synthesis, cell proliferation, apoptosis, and transcription [31,32]. Trx1 itself is regulated by oxidative stress conditions via binding of Nrf2 to an antioxidant responsive element (ARE) in the Trx promotor [33]. We observed that the expression of Trx1 increased by harmine treatment. Also, harmine plus HgCl₂ improved the decreased level of Trx1. We observed that Trx1 gene expression significantly increased following harmine treatment (p<0.001). In addition, compared with HgCl₂ group, expression of Trx1 significantly increased in the co-administered group (p<0.05).

Compared with 0.5 mg/kg HgCl₂ group, the Nrf2 gene expression significantly increased in harmine-only treatment group and 20 mg/kg harmine plus HgCl₂ group, p<0.001 and p<0.05, respectively.

Nrf2 is a transcription factor that is upregulated in response to oxidative stress and regulates transcription of genes encoding for cytoprotective enzymes and other proteins crucial for maintaining cellular homeostasis [29]. During oxidative stress, Nrf2 translocates to the nucleus and activates transcription of target genes such as NQO-1 and HO-1 [34]. Increased levels of oxidative stress caused by HgCl₂ might be attributed to insufficient ROS scavenging because of a failure in Nrf2 activation. However, we found that harmine could activate this antioxidant pathway, at the mRNA level. A number of stimuli like nitric oxide, heavy metals, cytokines, and modified lipids have been shown to induce HO-1 expression [35]. The activation of HO-1 appears to be an endogenous defensive mechanism applied by cells to reduce inflammation and tissue damage in hepatic
chemically induced injury [36]. Our results showed that harmine could ameliorate the negative effect of HgCl₂ on Ho-1 expression in liver tissue. Harmine administration alone increased the expression level of this gene. Combined treatment of harmine by HgCl₂ exposure exhibited a significant increase (p<0.05) in Ho-1 when compared with Hg-treated group. also, harmine significantly improved the expression of Ho-1 (p<0.001).

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 [37]. Nqo1 mRNA and protein expression and enzyme activity in mouse livers increased after bile duct ligation in wild-type mice, but not in Nrf2-null mice, a state that caused hepatocellular oxidative stress and injury [38]. A study on Nqo1-knockout mice revealed that this gene has a protective effect in acetaminophen-induced hepatotoxicity. In the absence of the role of Nqo1, acetaminophen triggered cell death in hepatocytes, severe mitochondrial dysfunction, and oxidative stress [39]. Previous studies have shown harmine could reduce oxidative stress, inflammation responses, and also apoptosis by suppressing the expression of nuclear factor kappaB (NF-κB), interleukin-1β (IL-1β), and caspase-1 [12,14]. In our study, Nqo1 gene expression significantly increased in 20 mg/kg harmine group (p<0.001). As expected, harmine-only resulted in a significant increase in Nqo1 expression level. The expression of Nqo1 was not significantly different for the co-administrated group compared to HgCl₂ group.

Although massive efforts are done in search for novel therapeutics against the mercury-induced toxicity, there is no effective treatment that completely eliminates the toxic effects [40]. Accordingly, the use of compounds having antioxidant properties and few apparent side effects may represent a helpful adjuvant strategy to eliminate the toxicity caused by organic and inorganic mercury forms [16,19,23,41,42].

Conclusion
In conclusion, the results of this study suggested that harmine has the potential to protect against mercury-induced hepatotoxicity. In addition, the mechanism of protection by harmine may be through modifying the enzymatic and non-enzymatic antioxidant levels. Based on these findings, it can be concluded that harmine can be a promising candidate for the management of liver mercury-intoxication.

Acknowledgments
This study was financially supported by Kermanshah University of Medical Sciences (Grant No., 980906).

Author contributions
Cyrus Jalili was involved in conceptualization, data curation, validation and funding acquisition; Sara Darakshian performed the analysis and prepared the original draft of the manuscript; Mohammadreza Azimi contributed in investigations, writing and editing of the manuscript; Ali Ghanbari contributed in project administration, supervision, writing and editing of the manuscript, validation and funding acquisition.

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.

References


Abbreviations
ALP: Alkaline phosphate; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CAT: catalase; GGT: gamma glutamyl transferase; GPx: glutathione
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peroxidase; H&E: hematoxylin and eosin; HO-1: heme oxygenase-1; IL-1β: interleukin-1β; LDH: lactate dehydrogenase; LPS: lipopolysaccharide; MDA: malondialdehyde; NF-Kb: nuclear factor kappaB; NO: nitric oxide; Nqo1: NAD(P)H quinone dehydrogenase 1; Nrf2: nuclear factor erythroid 2-related factor 2; RNA, ribonucleic acid; qRT-PCR: quantitative reverse transcription polymerase chain reaction; SOD: superoxide dismutase; TAC: total antioxidant capacity; Trx: thioredoxin