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Protective Effect of Silymarin on Mercury-Induced Acute Nephro-Hepatotoxicity in Rats

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Abstract: The present study was designed to investigate the detrimental effects of acute mercury intoxication on the liver and kidney and the probable alleviating capability of silymarin against such effects. Rats were divided into four groups. Group I control; group II rats were received a single subcutaneous (s/c) injection of mercuric chloride at dose of 5 mg/kg bwt; group III rats were orally given silymarin at dose of 200 mg/kg bwt/day for 7 days, then rats were injected s/c with a single dose of mercuric chloride (5 mg/kg bwt) and group IV rats were orally administered silymarin. Twenty four hrs after mercury injection all rats were euthanized. Mercury induced a significant increase in malondialdehyde (MDA) and a significant reduction in reduced glutathione (GSH) levels, significant changes in serum hepatic and renal function parameters (alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein, albumin, urea and creatinine. Mercury revealed marked degenerative and necrotic alterations in kidneys and liver. Silymarin returned MDA and GSH to the normal values, improved the mercury-induced serum biochemical changes of kidney and liver functions as well as histopathological alteration. Our results suggest that acute mercury intoxication induced marked nephro-hepatic deleterious effects which alleviated by silymarin pretreatment.

Key words: Mercury · Silymarin · Nephrotoxicity · Hepatotoxicity · Histopathology

INTRODUCTION

Mercury is a hazardous environmental and industrial pollutant which induces severe alterations in the body tissues of both humans and animals [1, 2]. The toxicity of mercury depends on the forms of the mercury compounds (elemental, inorganic and organic). Inorganic mercury accumulates predominantly in the kidneys [3] causing acute renal failure [4]. The uptake, accumulation and toxicity of inorganic mercury in the kidney have been related to its binding to endogenous thiol-containing molecules [5]. Thiol-containing enzymes have been recognized as the targets of inorganic mercury [3, 6]. Moreover, binding of mercuric ions to sulfhydryl groups may cause decreased glutathione levels, leading to increases in levels of reactive oxygen species (ROS), such as superoxide anion radicals, hydrogen peroxide and hydroxyl radicals [7], which provoke lipid, protein and DNA oxidation [8]. Considering that oxidative stress and endogenous thiol depletion are involved in inorganic mercury toxicity, it has been suggested that antioxidants could contribute to the treatment of mercury poisoning [9, 10]. In this way, melatonin [2, 11], curcumin [12] and vitamin E [13] have been found to play a protective effect against mercuric chloride ($HgCl_2$) induced acute renal toxicity. Similarly, a number of plant extracts with antioxidant properties have been shown to inhibit $HgCl_2$ induced renal toxicity [14-16]. Silymarin, a mixture of flavonolignans extracted from milk thistle plant (*Silybum marianum*) is a very strong antioxidant compound capable of scavenging both free radicals and ROS and thus it increases the antioxidant potential of cells by ameliorating the deleterious effects of free radical reactions [17]. In addition, as antioxidant, silymarin regulates the intracellular contents of the reduced glutathione (GSH) and chelates metal ions [18]. Silymarin

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is widely used for protection against various hepatobiliary disorders in Europe [19]. It is also reported that silymarin offers protection against chemical hepatotoxins such as CCl_4 [20, 21], acetaminophen [22], phalloidin, galactosamine and thioacetamide [23] and alcoholic liver diseases [24]. Recently, silymarin showed unconventional valuable activities as hypocholesterolemic and cardioprotective [25], antidiabetic [26], hypolipidemic [27], anti-inflammatory [20], neuroprotective and anti-apoptotic [28], anti-ageing [29] and nephroprotective [30-33] effects. Eser *et al*. [34] reported the protective effect of silymarin against cyclophosphamide-induced cystitis and bladder overactivity. Moreover, silymarin has been shown to be safe in animal models and no significant adverse reactions are reported in human studies [35, 36].

To the best of our knowledge, there are no studies concerning the nephroprotective effect of silymarin against mercury intoxication. Therefore, the present study was carried out to investigate (1) the adverse effect of acute mercury intoxication on the kidneys as well as liver based on serum biochemical parameters, oxidative stress and histopathological alterations and (2) The probable alleviating effect of silymarin against acute mercury intoxication in rats.

MATERIALS AND METHODS

Animals and Experimental Design: Twenty four adult male albino rats (weighing 180-200g) were obtained from a closed random bred colony at the Medical Research Institute of Alexandria University, Egypt. Animals were housed in cages with free access to the commercial basal food and water. The standard laboratory diet was purchased from Damanhur Feed Co. (Behera, Egypt). Rats were received humane care incompliance with the guidelines of the National Institutes of Health (NIH) of Animal Care and the local committee approved this study. After 2 weeks of acclimatization, all animals were randomly divided into four groups of six rats each: group I served as control and orally received 1 ml distilled water once daily for 7 days, then on the 7^{th} day and after 2 hrs from last dose of distilled water administration, rats were subcutaneously (s/c) injected with 0.5 ml normal saline at once. Group II (Hg-treated) rats were given a single s/c injection of mercury (Hg) in the form of mercuric chloride ($HgCl_2$, CHEMA TEC CO. Alexandria, Egypt) at dose of 5 mg/kg bwt (dissolved in normal saline) on the 7^{th} day of the experiment [37]. Group III (SIL + Hg-treated group) received orally 1ml of freshly prepared silymarin solution (dissolved in distilled water) at dose of 200 mg/kg bwt/day for 7 days [33, 38], then after 2hrs from last dose of silymarin administration rats were treated with Hg (dose same as that in the Hg-treated group). Group IV (SIL-treated) rats were orally administered silymarin solution (200 mg/kg bwt/day for 7 days); silymarin was kindly provided by Medizen Pharmaceutical plant Co. (Borg El-Arab, Egypt). Twenty-four hours after mercury injection, rats were anesthetized with ether then blood was collected from the inner canthus of the eye by heparinized capillary tube into clean dry test tube. The blood was centrifuged with 3000 rpm for 10 min. To separate the serum for assessment of some biochemical parameters. Immediately after the collection of blood samples, the animals were euthanized.

Serum Antioxidant Enzyme Activity and Oxidative Stress Assays

Reduced Glutathione (GSH): GSH was assayed by spectophotometric technique according to the method described by Sedlack and Lindsay [39]. Breifly, the method based on the reduction of 5,5 dithiobis (2-nitrobenzoic acid) with glutathione (GSH) to produce a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorption can be measured at 405 nm.

Lipid Peroxidation (LPO): LPO as malondialdehyde (MDA) was measured spectrophotometrically, after the reaction with thiobarbituric acid in acidic medium at 95°C for 30 min. To form thiobarbituric acid reactive product, the absorbance of the resultant pink product can be measured at 534 nm according to Placer *et al*. [40]. Using commercially available diagnostic kits (BIO DIAGNOSTIC Co. 29 Tahreer St., Dokki, Giza, Egypt).

Serum Biochemical Parameters: The activities of the serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were estimated according to Reitman and Frankel [41], alkaline phosphatase (ALP) using the method of Kind and King [42], total protein [43], albumin [44], urea [45] and creatinine [46].

Histopathological Examinations: After necropsy, tissue specimens of the liver and kidneys were collected and then rapidly fixed in 10% neutral-buffered formalin for at least 24 h. The fixed specimens were processed through the conventional paraffin-embedding technique [47], sectioned at 5 μm and stained with Mayer's hematoxylin and eosin (HE).

Statistical Analysis: Results were statistically analyzed by one-way analysis of variance followed by Duncan's multiple range test [48]. Data are presented as means plus or minus the standard error. The minimum level of significance was set at $P < 0.05$.

RESULTS

Serum Antioxidant Enzyme Activity and Oxidative Stress Assays: Compared to the control group, LPO was markedly increased in the Hg-treated rats as exhibited by a significant increase (P <0.05) in MDA level. SIL+Hg-treated and SIL-treated rats expressed normal levels of MDA in relation to the control rats (Table 1). Moreover, Table 1 shows that GSH level was significantly decreased (P <0.05) in the Hg-treated rats. Conversely, no significant alterations in GSH levels in SIL+Hg and SIL-treated rats.

Serum Biochemical Parameters: Table 1 shows that the Hg-treated and SIL+Hg-treated rats displayed a significant increase (P <0.05) in serum ALT, AST, ALP enzyme activities, urea and creatinine serum levels compared to the control. The elevation was less evident in the SIL+Hg-treated rats. There was a significant reduction in the total protein level in the Hg-treated and SIL+Hg-treated rats; however marked reduction was noticed in the Hg-treated rats. Serum albumin level was significantly decreased in the Hg-treated rats and insignificantly decreased in the SIL+Hg-treated rats. No significant changes in the globulin level of all groups. SIL-treated rats did not show any alterations in the biochemical parameters.

Histopathological Findings: Light microscope evaluation of kidneys in the control rats showed normal morphology of the renal parenchyma with well-defined glomeruli and tubules (Fig. 1A). Kidneys of Hg-treated rats after 24hrs of mercury injection showed severe diffuse acute necrosis of tubular epithelium, particularly at cortex and corticomedullary junction. The necrotic tubules exhibited fragmentation and shedding of tubular epithelium in their lumina (Fig. 1B). Moreover, dilatation of Bowman capsule, atrophy of glomerulus and degenerative changes were evident in (>75% of renal tubules) and represented by vacuolation of the epithelium of renal tubules and hyaline casts in their lumina. There were circulatory disturbances such as congestion of the intertubular blood vessels and interstitial edema. Conversely, pretreatment with silymarin improved the intensity of the lesions. Wherein, kidneys showed focal areas of degenerative and necrotic tubular changes, mostly confined to the renal cortex and corticomedullary junction (Fig. 1C). Also, mild congestion of the intertubular blood vessels and interstitial edema were present. Treatment of silymarin alone exhibited nearly normal structure of the renal tissues (Fig. 1D). Liver of Hg-treated rats showed moderate hepatocytic vacuolations, particularly at the periportal zones (Fig. 2B). There was dilatation of hepatic sinusoids with subsequent atrophy of hepatic cords. Moreover, focal areas of hepatic necrosis were evident. Pretreatment with silymarin ameliorated the harmful effect of mercury, since rats' liver showed nearly normal histological structure except for occasional hepatocytic vacuolations (Fig. 2C), few scattered individual cell undergoing necrosis as well as congestion of portal veins and dilated hepatic sinusoids. Livers of the control and SIL-treated rats revealed normal histoarchitecture (Fig. 2A, 2D).

DISCUSSION

Mercuric ion, one of strongest thiol-binding agents [49], increases the intracellular levels of reactive oxygen species and induces oxidative stress [50] resulting in tissue damage [51]. Toxicity of mercury is associated with superoxide radical generation and glutathione reduction [52, 53]. Our study demonstrates that the treatment of rats

Table 1: Effect of Silymarin (SIL), mercury (Hg) and their combination (SIL+Hg) on serum biochemical parameters of rats

	Parameter									
Group	ALT (u/l)	AST (u/l)	ALP (u/l)	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	Urea (mg/dl)	Creatinine (mg/dl)	MDA (nmol/dl)	GSH (mg/dl)
Control	15.5±0.87c	44.0±1.15c	112.5±1.44c	5.95±0.32a	4.05±0.09ab	1.90±0.23a	25.0±1.15c	0.37±0.03c	13.9±0.32b	5.60±0.23a
Hg-treated	38.0±1.73a	76.5±1.44a	224.0±16.17a	3.90±0.12b	2.10±0.23c	1.80±0.12a	56.0±0.58a	2.32±0.04a	24.0±1.01a	3.65±0.26b
SIL+Hg-treated	20.7±1.33b	56.7±3.84b	143.7±4.98b	4.67±0.27b	3.17±0.12b	1.50±0.15a	32.0±3.46b	0.85±0.08b	13.9±0.86b	5.13±0.20a
SIL-treated	16.0±1.15c	43.5±0.29c	112.0±0.58c	6.05±0.20a	4.20±0.53a	1.85±0.33a	24.5±1.44c	0.36±0.03c	13.7±0.95b	5.50±0.17a

Values are means ± standard errors

Means without a common letter differ significantly (P<0.05)

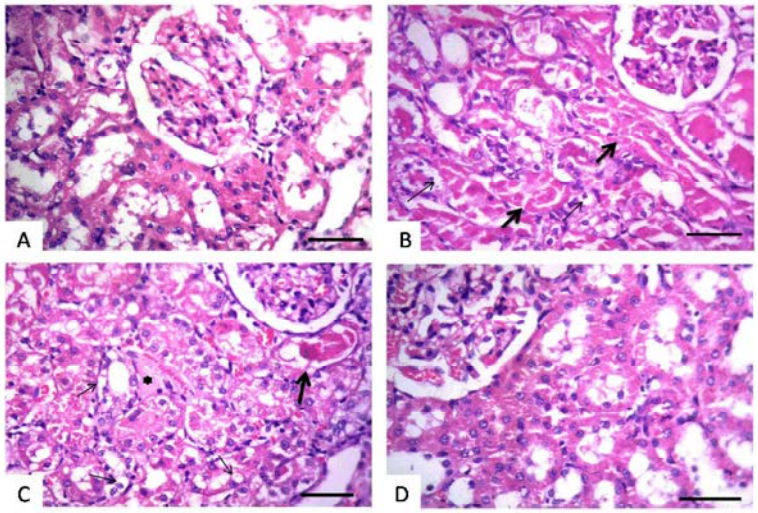

Fig. 1: Photomicrograph of kidney stained with HE (Bar=50 µm): (A) Normal morphology of the renal parenchyma with well-defined glomeruli and tubules, (B) Mercury-treated rats: Fragmentation and shedding of tubular epithelium in their lumina (thick arrows), vacuolar degeneration of tubular epithelial cells (thin arrows), (C) Silymarin + mercury treated-rats: Necrotic renal tubule (thick arrow), vacuolar degeneration of tubular epithelial cells (thin arrows), congestion of intertubular blood capillaries and interstitial edema (asterisk), (D) Silymarin-treated rats: Nearly normal histoarchitecture

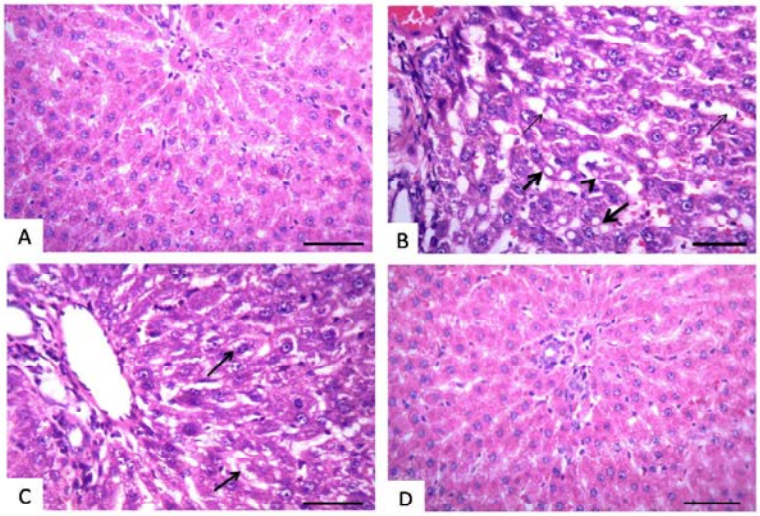

Fig. 2: Photomicrograph of liver stained with HE (Bar=50 µm): (A) Normal hepatic histoarchitecture, (B) Mercury-treated rats: Hepatic sinusoidal dilatation (thin arrows), periportal hepatocytes vacuolations (thick arrows) and focal area of hepatic necrosis (arrowhead), (C) Silymarin + mercury treated-rats: Occasional hepatocytic vacuolations (arrows), (D) Silymarin-treated rats: Nearly normal histoarchitecture

with $HgCl_2$ revealed a significant enhancement in MDA levels indicative of the generation of LPO. Enhanced lipid peroxidation levels were also reported in mercury toxicity by Sener *et al.* [54] and Agarwal *et al.* [13]. Mercuric chloride is known to increase the production of many reactive oxygen species (ROS) such as superoxide and H_2O_2 [55], which cause lipid peroxidation subsequently oxidative tissue damage [56, 57]. Glutathione, as both carrier of mercury and an antioxidant, has specific roles in protecting the body from mercury toxicity. GSH binds with mercury, forms a complex that prevents mercury from binding to cellular proteins and causing damage to both enzymes and tissue [58]. As a result of binding of mercury to glutathione and subsequent elimination of intracellular glutathione, levels of GSH are lowered in the cell and decrease the antioxidant potential of the cell. The present

study revealed that mercury-treated rats showed a significant depletion of serum GSH levels. Agarwal *et al.* [12, 13] reported a significant reduction of GSH levels in liver, kidney and brain tissues. Pretreatment with silymarin attenuated the Hg-induced oxidative damage. Hence, pretreatment with silymarin significantly restored the increased MDA and decreased GSH levels to the normal values. This could be attributed to the excellent antioxidant properties of silymarin [59, 60]. This property seems to be due to its ability to scavenge free radicals. The kidneys are the primary target organ for accumulation and toxicity of inorganic mercury [5]. In fact, during as little as 1 hour, 50% of an administered dose of inorganic mercury is present in the kidney [61]. Within the kidney, the majority of mercuric ions were detected in the cortex and outer stripe of the outer medulla. This finding was expected considering that the proximal tubule, which spans these two renal zones, is the primary site of accumulation of mercuric ions [5]. Our histopathological findings in the kidney tissue of Hg-treated rats revealed: severe diffuse acute necrosis of tubular epithelium, particularly at cortex and corticomedullary junction, fragmentation and shedding of tubular epithelium in the lumina of the renal tubules and interstitial edema as a result of tubular leakage. The interaction of mercury with protein sulfhydryl groups is thought to play an important role in nephrotoxicity induced by mercury at cellular level [5]. Changes in mitochondrial morphology and function are very early event which follow mercuric chloride administration, which suggests that mitochondrial dysfunction and oxidative stress have an important role in mercury induced renal toxicity. Our findings were similar to those reported by Al-Saleh *et al.* [62], Alam *et al.* [16], Sharma *et al.* [63], Augusti *et al.* [37] and Agarwal *et al.* [12, 13]. These results were supported by enhanced serum creatinine and urea levels in mercury-treated rats that indicate marked nephrotoxicity [64]. Pretreatment with silymarin protected against Hg-induced nephrotoxicity. This was manifested by deceased serum creatinine and urea levels and diminished the intensity of the renal lesions. The nephroprotective effect of silymarin was reported by Karimi *et al.* [30], El-Shitany *et al.* [31], Abdelmeguid *et al.* [32] and Soto *et al.* [33]. Mercury intoxication showed a significant increase in AST, ALT and ALP activities [65]. These results may be due to hepato cellular necrosis or membrane damage which causes the release of these enzymes into the circulation [66]. Furthermore, significant reduction in the serum total protein and albumin levels may be due to decline in protein synthesis by hepatic cells reflecting the hepatic dysfunction that accompanied by mercury treatment [65]. These results are supported by the histopathologic changes observed in the liver: periportal hepatocytic vacuolations, dilatation of hepatic sinusoids with subsequent atrophy of hepatic cords and focal areas of hepatic necrosis. Similar changes were also reported by Al-Saleh *et al.* [62] and Agarwal *et al.* [12, 13] in liver tissue after mercury exposure. Pretreatment with silymarin significantly reduced the activities of the above marker enzymes in mercury-treated rats. This indicates that silymarin tends to lessen liver damage by maintaining the integrity of the plasma membrane, thereby suppressing the leakage of enzymes through membranes, exhibiting hepatoprotective activity [67].

In conclusion, this study demonstrated the detrimental effects of mercury induced acute toxicity on the kidneys as well as liver. Additionally, silymarin ameliorated Hg-induced nephrotoxicity and protected against Hg-induced hepatotoxicity in male albino rats.

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