Mercury (Hg) is naturally distributed throughout the environment. Anthropogenic activities and industrialization have increased the release of Hg to the environment (1). Since mercury is widely used in agriculture, medicine, industrial manufacture, it is impossible to avoid being exposed to some forms of mercury (2). Mercury is found in three forms (elemental, inorganic and organic) and all of them have toxic effects (3). Mercury intoxication can occur during the environmental, occupational or accidental exposure. The toxicity of mercury depends on the dose, route and duration of exposure (4). The liver plays an important role in mercury biotransformation and metabolism. Mercury localizes in the liver tissue after exposure to its compounds (5, 6). Mechanism of Hg-induced hepatotoxicity is not completely understood, but in many studies has been found that mercury induced oxidative stress is one of the major molecular mechanisms, damaging the liver (3). Mercuric chloride toxicity causes overproduction of reactive oxygen species (ROS) in liver, leading to oxidative damage and cellular dysfunction (1). Regarding the non-negligible oxidative stress in mercury induced hepatotoxicity, antioxidants are expected to have a protective role against it (7). Recently, there is overwhelming attention to herbal products and natural antioxidants for treatment and prevention of many diseases compared to synthetic antioxidants (2, 8). Pomegranate (Punica granatum L.) from Punicaceae family is native to the region from northern India to Iran. It is also widely cultivated in parts of Southwest America, California, Arizona and Africa (9). Pomegranate has extensively been used in folk medicine for various purposes. Recently, studies have shown that pomegranate has several pharmacological activities such as antimicrobial (10, 11), antioxidant, anti-inflammatory and anticarcinogenic effects (12). Pome-
granate derived products have shown beneficial effects on the treatment and prevention of various diseases such as cancer, cardiovascular disease, neurological disorders, diabetes etc. (13, 14). PSO comprising 12-20% of total seed weight consists of approximately 80% conjugated octadecatrienoic fatty acids mainly punicic acid (9cis, 11trans, 13cis acid). Also, catalpic acid (C18:3-9trans, 11trans, 13cis) and α-eleostearic acid (C18:3-9cis, 11trans, 13trans) are isomers of conjugated linolenic acids (CLnAs) found in PSO. The oil is characterized by a high content of fatty acids, of which 99% are triglycerides. The fatty acid composition of PSO obtained from specific varieties of pomegranate has been reported (punicic acid, linoleic acid, oleic acid, stearic acid, palmitic acid, others) (15-18). Sterols, steroids, cerebrosides, lignins, hydroxycinnamic acids and the potent antioxidant lignin derivatives are considered as minor components of the oil. The oil consists of high contents of phytosterols such as β-sitosterol, campesterol, stigmasterol and tocopherols (α and γ-tocopherol) (12, 19). Although some published reports demonstrated the protective effects of pomegranate seed oil (PSO) against atherosclerosis and nephrotoxicity (20-22) but there is no evidence concerning the hepatoprotective effects of PSO against HgCl2-induced acute toxicity. Therefore, the present study was designed to evaluate the protective effects of PSO against liver damage induced by i.p. injection of HgCl2 in rat.

MATERIALS AND METHODS

Chemicals
Mercuric chloride (HgCl2) was obtained from May & Baker company (England), TMP (tetra-methoxypropane), Trizma base (Tris (hydroxymethyl) aminomethane), TBA (2-thiobarbituric acid), n-butanol , DTNB (2,2’-dinitro-5,5’-dithiobenzoic acid), NaOH (sodium hydroxide), phosphoric acid, HCl (hydrochloric acid), Na2EDTA (ethylenediaminetetraacetic acid disodium salt), KCl (potassium chloride) and diethyl ether were purchased from Merck Co. (Darmstadt, Germany). Pomegranate seed oil (d = 0.81 g/mL at 25°C) was a kind gift from Urom Narin Co. (Uromeya, I. R. Iran).

Animals
The study was performed on 24 adult male W/A rats with 170–190 g body weight (Animal House, School of Medicine, Mashhad, Iran). The animals were housed in a humidity and temperature controlled environment and maintained on a light-dark cycle of 12 h. They were allowed free access to standard laboratory diet and water ad libitum. All experimental procedures were conducted in accordance with the university ethics committee and were conducted under national laws and the National Institutes of Health guidelines for the use and care of laboratory animals.

Experimental design
After acclimatization, animals were randomly divided into five groups (six each), Group 1 (control group) and 2 received corn oil (1 mL/kg) and PSO (0.8 mL/kg) for 3 days, respectively. Group 3 was injected a single dose of HgCl2 (with the concentration of 5 mg/kg body weight in 0.9% NaCl). In group 4 and 5, PSO were administered 0.4, 0.8 mL/kg, one hour before HgCl2 (5 mg/kg) injection for 3 consecutive days. All injections were performed intraperitoneally. Twenty four hours after last HgCl2 injection, all rats were anesthetized with ether. Blood was directly collected from heart, centrifuged at 1000 × g for 15 min to separate the serum for assessment of some biochemical parameters. Rat’s liver were then removed, the piece of liver was homogenized in cold KCl solution (1.5%, pH = 7) to give a 10% homogenate suspension for biochemical assays. The other piece was fixed in 10% formalin and sectioned for histopathological studies.

Biochemical study
Serum ALT and serum AST
ALT and AST level measurement was according to the International Federation of Clinical Chemistry (IFCC) method and was expressed as units per liter (23).

Lipid peroxidation (LPO)
MDA (malondialdehyde) level is identified as a main products of lipid peroxidation that reacts with TBA to give a red color species (thiobarbituric acid reactive substance (TBARS)), which have peak absorbance at 532 nm. Half milliter of homogenate in centrifuge tube was mixed with 3 mL phosphoric acid (1%) and 1 mL TBA (0.6%). Then, all tubes were placed in a boiling water bath for 45 min. The tubes were then cooled and 4 mL of n-butanol was added to the reaction mixture, vortexed for 1 min, and centrifuged at 60,000 × g for 20 min. The absorbance of supernatant was determined at 532 nm. MDA content was expressed as nanomoles per gram of tissue.

MDA concentration of the liver samples were calculated using the standard curve of MDA (concentration range of 0–40 μM) (24).
Total sulfhydryl (SH) groups assay

The sulfhydryl group content in liver was measured spectrophotometrically using DTNB (2,2'-dinitro-5,5'-dithiobenzocic acid) as a coloring reagent. This reagent produces a measurable yellow-colored product when it reacts with sulfhydryl group.

Fifty µL of homogenate was mixed with 1 mL Tris-EDTA buffer (pH = 8.6) in test tube and its absorbance was read at 412 nm (A1). Twenty µL of 10 mM DTNB was added to the mixture. After 15 min at laboratory temperature, the absorption was measured again (A2). Blank (B) was the absorbance of DTNB reagent. Total SH groups are calculated using an equation (25):

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\text{Total thiol concentration (mM)} = \frac{(A2 - A1 - B) \times 1.07}{0.05} \times 13.6
\]

Histological study

Liver tissue samples were fixed in 10% formalin for at least 24 h. The fixed specimens were processed, using paraffin-embedding technique and hematoxylin and eosin (H&E) staining was performed for histopathological examinations through the light microscope (7).

Statistical analysis

The values are expressed as the mean ± SEM. All statistical analyses were performed using Prism 5 software. Data were analyzed using one-way analysis of variance followed by Tukey-Kramer post-hoc test for comparison between groups. The minimum level of significance was set at p < 0.05.

RESULTS

Biochemical studies

Serum ALT, AST enzyme activity

The activities of serum ALT, AST after mercuric chloride exposure and PSO pretreatment (0.4, 0.8 mL/kg) are shown in Figures 1 and 2. HgCl2 intoxicated rats displayed a significant elevation in serum ALT enzyme activity, compared to the control group (p < 0.01). Pretreatment of PSO (0.8 mL/kg) caused a significant decline in ALT activity as compared to HgCl2 intoxicated animals. Also HgCl2 injection caused significant elevation in AST level as compared with the control group (p < 0.05). Rats, which were pretreated with PSO (0.8 mL/kg), exhibited a significant reduction in AST serum level with respect to HgCl2 group (p < 0.05) (Figs. 1, 2).

Figure 1. Effect of the pomegranate seed oil pre-treatment against HgCl2 intoxication on serum ALT activity. Values are expressed as the mean ± SEM (n = 6). * p < 0.05, ** p < 0.01 as compared with HgCl2-treated group

Figure 2. Effect of the pomegranate seed oil pre-treatment against HgCl2 intoxication on serum AST activity. Values are expressed as the mean ± SEM (n = 6).* p < 0.05 as compared with HgCl2-treated group; # p < 0.05 as compared with control group
However, AST levels in rats pretreated with PSO (0.8 mL/kg) showed no significant difference when compared with the control group, but there was a significant difference in AST levels between the control group and the PSO (0.4 mL/kg) pretreated rats (p < 0.05). Rats treated with PSO (0.8 mL/kg) alone did not show significant differences in AST and ALT levels when compared with the control group.

Lipid peroxidation

The lipid peroxidation (LPO) level in liver is expressed as malondialdehyde levels (MDA). As shown in Figure 3, mercury chloride administration significantly increased tissue level of malondialdehyde compared to control group (p < 0.001). Compared with the HgCl₂ group, MDA levels decreased significantly in the PSO (0.8 mL/kg) pretreatment group (p < 0.01). However, the rats pretreated with PSO (0.4, 0.8 mL/kg) showed significant differences in MDA levels when compared with the control group (p < 0.001, p < 0.05).

Total thiol content

Figure 4 shows that HgCl₂ treatment decreased the total thiol content significantly in the rat liver homogenates (p < 0.001). PSO pretreatment (0.8 mL/kg) exhibited a significant elevation in total thiol concentrations, as compared with HgCl₂ group (p < 0.01). However, significant differences were observed in the total thiol content between the PSO (0.4, 0.8 mL/kg) pretreated rats and the control group (p < 0.001, p < 0.01).

Rats treated with PSO (0.8 mL/kg) alone did not show significant differences in lipid peroxidation and the total thiol content, as compared with the control group (Figs. 3, 4).

Histopathological observations

Histopathological analyses showed focal necrosis of hepatocellular, inflammatory cell infiltration and cytoplasmic vacuolization in the HgCl₂-treated rat liver sections (Fig. 5). Liver section of rat, pre-treated with PSO, showed partial recovery and very small hepatocellular necrosis (Fig. 5).

DISCUSSION

Mercury as a toxic transition metal, induces oxidative stress through overproduction of ROS (25). ROS cause damage to cellular proteins, nucleo-
Acid, and lipids, leading to cell membrane damage and cellular dysfunction (3). The liver is a major site involved in mercury metabolism, hence, resulting in mercury accumulation in the liver (1). Results of the present study showed a significant increase in activity of serum transaminases (AST and ALT) after HgCl₂ treatment. There are many reports indicating that activity of these enzymes were significantly elevated in rat, given HgCl₂ (5, 7). This may be due to hepatocellular necrosis, which causes the release of these enzymes into the blood circulation after cellular damage and rupture of the plasma membrane (2). The biochemical findings were also confirmed by histopathological changes after HgCl₂ injection. These changes were mostly in hepatocellular necrosis, vacuolations and inflammatory cell infiltration. Similarly, Sharma et al. (2) and Kumar et al. (26) reported that mercuric chloride induces centrilobular necrosis and cytoplasmic vacuolization in liver. Oda and El-Ashmawy (7) showed that HgCl₂ caused hepatic injury characterized by periportal hepatocytic vacuolations and hepatic necrosis in rats. Also Deng et al. (1) observed several histopathological changes such as necrosis after HgCl₂ exposure. Lipid peroxidation (LPO) plays a crucial role in HgCl₂-induced hepatotoxicity (26). LPO can destroy biological membranes and alter cell membrane permeability (27, 28). MDA is the main product of LPO and considered as a marker of oxidative damage (29). The present study revealed that administration of HgCl₂ caused significant enhancement in LPO level as expressed by increased tissue levels of MDA. Similar results were reported by Oda and El-Ashmawy (7) and Deng et al. (1). The increased LPO level could be due to the overproduction of free radicals and decreased antioxidant enzyme activities which cause oxidative tissue damage (6).

Sulphydril groups are highly-reactive constituents of non-protein and protein molecules such as catalytic or binding domains of enzymes, and they play important roles in several metabolic and biochemical processes such as detoxification mechanisms, maintenance of protein systems and activation of enzymes including antioxidant enzymes (superoxide dismutase, catalase, etc.). They can scavenge oxygen-derived free radicals (5, 22, 30). There are several reports suggesting that mercury exerts toxic effects mainly through the formation of complexes with sulphydryl groups and depletion of intracellular thiol pool, which may induce oxidative stress (2, 5, 31). The results revealed significant decrease of total sulphydryl groups in HgCl₂-treated rats. It was observed that pretreatment with PSO significantly increased total sulphydryl group levels.
Deng et al. (1) found that overproduction of ROS in the liver was caused by HgCl₂ exposure and oxidative stress is probable mechanism for HgCl₂-induced hepatic damage.

Studying the section of hepatic tissue following administration of PSO revealed a milder lesion in liver samples of PSO treated rat compared to rats treated with HgCl₂ alone. On the other hand, treatment with PSO was found to suppress (p < 0.05) the elevation of serum AST and ALT activities induced by HgCl₂ treatment in rats. This finding implies that PSO play a protective role in liver tissue against HgCl₂ injury. It may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity. In several studies, PSO administration led to significant reduction of MDA concentration in tissue homogenate samples (5, 21, 24). PSO indicated antioxidative properties by reduction in the MDA levels and the enhancement of decreased glutathione (20). PSO administration may decrease HgCl₂ induced hepatotoxicity by quenching these toxic metabolites. PSO did not exert any significant alteration when administered alone but significantly reduced or normalized the alterations caused by HgCl₂ injection. Pomegranate seed oil, consists of high content of conjugated fatty acids among which punicic acid is the most common. Other components of pomegranate seed include γ-tocopherol, ursolic acid, sterols (daucosterol, campesterol, stigmasterol, β-sitosterol), hydroxynaphthoic acids (gallic and ellagic), coniferyl 9-O-[β-D-apiofuranosyl(1-6)]-O-β-D-glucopyranoside, sinapyl 9-O-[β-D-apiofuranosyl(1-6)]-O-β-D-glucopyranoside (12, 32) which showed antioxidant activity (32). Moreover, it contains polyphenolic compounds with antioxidant and anti-inflammatory activity (22, 33). Several studies reported anti-cancer and anti-inflammatory effects for these components: e.g., hydroxybenzoic acids cause inhibition of growth and induce apoptosis in human DU-145 prostate cancer cells (34) and sterols inhibit pro-inflammatory cytokine production in mice (35). PSO contains ellagic acid, an antioxidant compound that removes peroxyl radicals and prevents lipid peroxidation induced by Cu²⁺ (36). According to the presence of a variety of biologically active compounds in PSO, its antioxidant and free-radicals scavenging properties may be partially responsible for its hepatoprotective activity.

CONCLUSION

In the present study, HgCl₂ administration induced hepatic damage which was alleviated by PSO pretreatment. Oxidative stress may be one of the most important mechanisms of HgCl₂ induced hepatic injury and dysfunction. Our data suggest that PSO has protective and preventive effects against oxidative damage in the liver of HgCl₂-treated rats.

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