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Rutin ameliorates mercuric chloride-induced hepatotoxicity in rats via interfering with oxidative stress, inflammation and apoptosis

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ABSTRACT

Objective: Mercury is a global environmental pollutant and is responsible for several organ pathophysiology including oxidative stress-induced liver disorders. Therefore, the present study was conducted to evaluate the potential ameliorative effects of rutin on mercury chloride ($HgCl_2$)-induced hepatotoxicity in adult male rats.
Methods: $HgCl_2$ was intraperitoneally injected at a dose of 1.23 mg/kg body weight for 7 days alone or in combination with the orally rutin (50 and 100 mg/kg body weight).
Results: Rutin treatment significantly improved liver function tests [alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT)], and increased activities of antioxidant defense system [catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx)] and glutathione (GSH) content. The histological alterations and epidermal growth factor receptor (EGFR) expression in the $HgCl_2$-induced liver tissues were decreased by administration of rutin. Furthermore, rutin reversed the changes in levels of apoptosis and inflammation related proteins involving p53, Bcl-2 associated X protein (Bax), B-cell lymphoma-2 (Bcl-2), cytochrome c, nuclear factor kappa B (NF-κB), tumor necrosis factor-α (TNF-α), B-cell lymphoma-3(Bcl-3) and interleukin-1β (IL-1β), and inhibited p38α mitogen-activated protein kinase (MAPK) and cysteine aspartate specific protease-3 (caspase-3) activations.
Conclusion: The data of the present study suggest that rutin effectively suppress $HgCl_2$-induced hepatotoxicity by ameliorating oxidative stress, inflammation and apoptosis.

1. Introduction

Mercury is a highly toxic, redox-active element which represents one of the main agents responsible for environmental pollution [1]. Mercury was recorded as the third most dangerous heavy metal after arsenic and lead, according to the Agency for Toxic Substance and Disease Registry Agency (ATSDR) [2]. It can be found in three different chemical forms; elemental mercury (Hg^0), organic mercury (mainly methylmercury), and inorganic mercury (mainly mercuric chloride) [3].

Mercury chloride ($HgCl_2$) is one of the most toxic forms of mercury, because it crosses biological membranes and easily forms organo-mercury complexes with proteins [4]. Based on the available experimental data, the accumulation of $HgCl_2$ in the liver induces oxidative stress, depletion of glutathione, and mitochondrial depolarization, due to interference of intracellular mercury with enzyme functions, disturbing ATP production and protein synthesis [4,5]. $HgCl_2$ also causes to undermine the antioxidant defense systems by reacting with cellular thiols [6]. In addition, $HgCl_2$ is reported as a potent apoptosis inducer, through cytochrome c release, activation of p38 mitogen-activated protein kinases (MAPK) and increase in nuclear factor kappa-B (NF-κB) level [7]. Numerous toxicological and biological studies reported that $HgCl_2$ caused neurotoxic [8], hepatotoxic [9], nephrotoxic [10], genotoxic [11] and hematotoxic [3] effects in experimental animals. In the clinic, chelating agents are used for medical treatment of mercury toxicity. The chelators used for this purpose form chelation compounds with toxic metal ions, thus eliminating them from the body easily through the excretory system. However, these treatments show some extent of toxicity and are ineffective in repairing tissue damage [7,9]. Considering that inorganic mercury compounds supress antioxidant

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defense system and induces apoptosis, natural antioxidant products have been proposed as potential therapies with non-toxic and protective effects.

Flavonoids are plant secondary metabolites commonly found in the vegetables and fruits regularly consumed by humans [12–16]. Flavonoids present biological and pharmacological properties such as antioxidant, antihepatotoxic, antimicrobial, anti-apoptotic, antitumoral, anti-inflammatory, antidiabetic, anti-allergic and anticholinergic [17–22]. Rutin (quercetin 3-rhamnosylglucoside or sophorin) is a glycone of quercetin having a flavonol structure [23,24]. It is abundantly found in buckwheat seed, passion flower, leaf of tomato, wine, onions, apples and tea [23,25]. Rutin exhibits multiple pharmacological properties such as antioxidant, antiallergic, antibacterial, antiulcer, anticarcinogenic, anti-inflammatory, anti-diabetic, antimutagenic, and potent scavenger of superoxide radicals [24,26]. It has been reported that rutin prevented methotrexate-induced hepatotoxicity in a rat model [27].

To the best of our knowledge, there is no such report or data related to the protective effects of rutin against $HgCl_2$-induced hepatotoxicity. Therefore, the present study aimed to investigate the ameliorative effect of rutin against $HgCl_2$-induced hepatotoxicity in male rats and to elucidate the underlying molecular mechanism.

2. Material and methods

2.1. Chemicals and reagents

Rutin (≥94%), $HgCl_2$ (≥99.5%) and all other reagents were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). All chemicals and reagents were of the highest purity grade. Alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) assay kits were supplied by TML, Diagnostic Medical Products, (Ankara, Turkey). Antibodies for immunohistochemistry and western blot were supplied by Santa Cruz Biotechnology (USA). B-cell lymphoma-3 (Bcl-3), tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), NF-κB and p53 assay kits were purchased from Sunred Biological Technology (Shanghai, China). p38α mitogen-activated protein kinase (p38α MAPK) assay kit were obtained Elabscience Biotechnology, (China).

2.2. Animals and experimental design

Male Sprague Dawley rats, weighing approximately 250–270 g, were purchased from the Experimental Research and Application Center, Ataturk University (Erzurum, Turkey). The animals were kept in polypropylene cages under standard laboratory conditions of relative humidity (45 ± 5%), temperature (25 ± 2 °C) and 12 h light/dark cycle and were provided food pellets and tap water *ad-libitum*. The study protocol was approved by the Ethical Committee for Animal Experiments, Ataturk University (Approval no: 2016-8/161).

Adult male rats (n = 35) were randomly divided into 5 groups (7 rats/group). The toxic dosage of $HgCl_2$ has been determined (sub-lethal dose of $HgCl_2$ 1.23 mg/kg b.w.) by Bharathi and Jagadeesan [28]. And it has sufficient to elicit mild or moderate oxidative stress in rats.

Group I (Control); Only vehicle intraperitoneal (i.p.) saline was given to animals for 7 days.

Group II (Rutin 100); the animals were administered orally rutin (100 mg/kg b.w.) for 7 days [24].

Group III ($HgCl_2$); the animals were administered $HgCl_2$ (1.23 mg/kg b.w.) in physiological saline i.p. for 7 days.

Group IV ($HgCl_2$ + rutin 50); the animals were administered orally rutin (50 mg/kg b.w.) 30 min after the injection of $HgCl_2$ (1.23 mg/kg b.w.) administration for 7 days.

Group V ($HgCl_2$ + rutin 100); the animals were administered orally rutin (100 mg/kg b.w.) 30 min after the injection of $HgCl_2$ (1.23 mg/kg b.w.) administration for 7 days.

All animals were exposed to sevoflurane anesthesia and were killed by decapitation 24 h after the last treatment. Blood samples were collected from all groups and serum separation was performed at 3000 rpm for 10 min. Serum was stored at − 20 °C until analysis. The liver tissues were dissected and used for biochemical, molecular and histopathological examinations.

2.3. Determination of liver function markers

Serum ALT, AST and ALP enzyme activities were determined in an ELISA reader (Bio-Tek, Winooski, VT, USA) with commercial kits according to the manufacturer's instructions.

2.4. Determination of lipid peroxidation and antioxidant defences

The liver tissues were homogenized in a homogenizer (Tissue Lyser II, Qiagen, Netherlands) using a buffer of 1.15% potassium chloride to obtain a 1:10 (w/v) homogenate. For malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) assays, homogenates were centrifuged for 15 min at 3500 rpm at +4 °C. To assay glutathione (GSH) level and glutathione peroxidase (GPx) activity of liver homogenates, they were centrifuged for 20 min at 10,000 rpm at +4 °C. After centrifugation, the obtained supernatants were subjected to assays as soon as possible. The levels of MDA (indicator of lipid peroxidation) in liver tissues, were assessed following the methods of Placer, Cushman and Johnson [29]. Reduced GSH content was determined by the method of Sedlak and Lindsay [30]. GPx activity was measured by the method of Lawrence and Burk [31]. SOD activity was assayed by the method of Sun, Oberley and Li [32]. CAT activity was measured by the method of Aebi [33]. Protein content was measured by the method of Lowry, Rosebrough, Farr and Randall [34]. Biochemical analyzes were measured by ELISA Plate Reader (Bio-Tek, Winooski, VT, USA).

2.5. Determination of inflammatory markers

NF-κB, Bcl-3, IL-1β and TNF-α levels were estimated in the liver tissue homogenates using commercially available kits. Absorbance was read at 450 nm. The contents of these parameters were expressed as ng/g tissue. Tissue preparation for ELISA kits was performed as described in our previous studies [35,36].

2.6. Determination of p53 levels and p38α MAPK activities

Levels of p53 protein and p38α MAPK activities in the liver tissues was detected with a commercial rat ELISA kit according to the manufacturer's instruction. Absorbance was read at 450 nm.

2.7. Western blotting analysis

Tissues from the treated (experimental) rats were homogenized on ice in a 1:5 (w/v) Mammalian Cell Extraction Kit (ab65399, Abcam, UK) and the mixture was centrifuged at 14,000 rpm for 3 min. Protein concentrations were loaded equal amounts of protein to the wells during the experimental stages. Protein samples were made ready for experiments with Laemmli sample buffer (Tris-HCl pH: 6.8, Glycerol, bromophenol blue, sodium dodecyl sulfate, 2-mercaptoethanol). Equal concentrations of protein samples were then run to electrophoresis on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins separated in the gel were transferred to nitrosellulose membranes (0.2 μm pore size). Nitrosellulose membranes were washed 2 times in TBS-T (TBS-0.05% Tween-20) for 5 min and blocked for 1 h prior to the application of primary antibody in 5% skimmed milk powder. The primary antibodies Bax (1:1000 dilution, sc-20067), Bcl-2 (1:1000 dilution, sc-7382), cytochrome c (1:1000 dilution, sc-13156), procaspase-3 (1:500 dilution, sc-271759) and β-Actin (1:500 dilution, sc-47778) were prepared in 5% skimmed milk powder.

Table 1
The effects of rutin on biochemical parameters in $HgCl_2$-induced hepatotoxicity in rats.

Parameters	Control	Rutin 100	$HgCl_2$	$HgCl_2$ + Rutin 50	$HgCl_2$ + Rutin 100
AST (U/L)	56.67 ± 0.94[a]	53.85 ± 0.92[a]	138.55 ± 1.58[d]	109.25 ± 2.34[c]	81.95 ± 1.15[b]
ALP (U/L)	66.51 ± 1.46[a]	61.40 ± 0.76[a]	167.63 ± 2.05[d]	127.03 ± 2.25[c]	85.61 ± 1.37[b]
ALT (U/L)	36.94 ± 0.82[a]	35.01 ± 0.73[a]	109.79 ± 2.32[d]	64.34 ± 2.18[c]	49.05 ± 1.01[b]
MDA (nmol/g tissue)	45.77 ± 0.65[b]	42.53 ± 0.85[a]	94.39 ± 0.96[e]	64.95 ± 0.79[d]	53.38 ± 0.87[c]
SOD (U/g protein)	40.89 ± 0.60[a]	41.91 ± 0.58[a]	21.03 ± 0.71[d]	31.54 ± 0.58[c]	33.84 ± 0.52[b]
CAT (katal/g protein)	53.36 ± 0.82[a]	55.02 ± 0.42[a]	32.14 ± 0.61[c]	43.55 ± 0.87[b]	44.54 ± 0.78[b]
GPx (U/g protein)	42.58 ± 1.12[a]	44.30 ± 0.57[a]	22.47 ± 0.79[d]	31.20 ± 0.68[c]	34.20 ± 0.77[b]
GSH (nmol/g tissue)	5.97 ± 0.31[b]	6.58 ± 0.09[a]	2.38 ± 0.13[e]	3.83 ± 0.07[d]	4.99 ± 0.07[c]

Values are expressed as mean ± SEM of seven rats in each group. Different superscripts (a–e) in the same row indicate significant difference ($p < 0.05$) among groups. (AST; aspartate aminotransferase, ALP; alkaline phosphatase, ALT; alanine aminotransferase, MDA; malondialdehyde, SOD; superoxide dismutase, CAT; catalase, GPx; glutathione peroxidase, GSH; glutathione).

Nitrosellulose membranes were incubated with primary antibodies at 37 °C for 3 h. The washing process of the blots was carried out in TBS-T for 3 times for 5 min. The membranes were then incubated with secondary antibody (horseradish peroxidase-conjugated goat anti mouse IgG, sc-2005) at 37 °C for 1 h 30 min. Protein concentrations were developed on medical films using ECL (Advansta, CA) buffer to detect specific binding. Protein levels were analyzed densitometrically with an image analysis system (GelDoc EZ and Image Lab. 5.2.1 software, Bio-Rad, USA).

2.8. Histopathological examination

For histopathological examination, the livers of the animals that were taken into autopsy were dissected. The liver tissue was fixated for 48 h in a 10% neutral buffer formalin. The fixated tissues were subjected to routine tissue follow-up. After the follow-up, the tissues were embedded in paraffin blocks. Cross-sections of 4 μm thickness were obtained from each block. After the cross-sections that were put on slides were left in a stove for 1 h, the preparates were histologically stained by hematoxylin & eosin (H&E). The preparates that were stained were observed under a light microscope (Olympus BX51, Germany). The samples were evaluated based on their immunopositivity as none (-), mild (+), moderate (+ +) and severe (+ + +).

2.9. Immunohistochemical examination

From the tissues embedded in paraffin blocks, cross-sections were put onto adhesive-containing (poly-L-Lysin) slides. The cross-sections were passed through gradients of xylol and alcohol, and deparaffinization and dehydration were performed. The tissues were washed with phosphate-buffered saline (PBS), kept for 10 min in the 3% H_2O_2 solution prepared with methanol, and endogenous peroxidase inactivation was performed. To prevent the antigens in the tissues from being masked, the samples were microwave-treated for 2 x 5 min with an antigen retrieval solution. For the antigen-antibody reaction, the tissues were incubated for 60 min in primary antibody [Epidermal growth factor receptor (EGFR)] (catalog no: sc-373746) at 37 °C. The following procedures were carried out based on the instructions of the immunohistochemistry kit (Abcam HRP/DAB Detection IHC kit). In order to make the immune complex visible, 3-3′ Diaminobenzidine (DAB) chromogen was used. The preparates that were counterstained by hematoxylin were observed under a light microscope. The samples were evaluated based on their immunopositivity as none (-), mild (+), moderate (+ +) and severe (+ + +).

2.10. Statistical analysis

The biochemical data were analyzed using version 20.0 of the computer-based statistical product and service solutions (SPSS, Chicago, IL). Data were expressed as mean ± SEM. ANOVA and Tukey's post hoc tests were used for comparison between groups. A level of $p < 0.05$ was defined as statistically significant. For Western blotting, statistical analysis was performed by One-way ANOVA, Newman-Keuls Post-Hoc Test in GraphPad Prism5 software; $p < 0.05$ was considered as statistically significant.

The Kruskal–Wallis test was used for analyzing semi-quantitative histopathological data. If significant differences were revealed, the Mann Whitney U test was used for two-way comparisons. The SPSS 13.0 package was used for these statistical analyses.

3. Results

3.1. Rutin decreases the activities of hepatic injury markers in the serum

Serum AST, ALP and ALT enzyme activities did not change in control and only rutin 100 groups (Table 1). Treatment with $HgCl_2$ caused a significant increase in the activities of these enzymes compared to the control and only rutin 100 groups ($p < 0.05$). In contrast to this, the activities of AST, ALP and ALT in the $HgCl_2$ + rutin 50 and $HgCl_2$ + rutin 100 groups were significantly lower than that of only $HgCl_2$ intoxicated group ($p < 0.05$).

3.2. Rutin increases the activities of antioxidant enzymes in liver

The effects of rutin and $HgCl_2$ treatments on enzymatic activities of SOD, CAT and GPx in liver tissue are given in Table 1. The data showed that treatment with $HgCl_2$ caused a significant decrease ($p < 0.05$) in the activities of SOD, CAT and GPx in rat liver. Rutin (50 and 100 mg/kg b.w.) administrated in $HgCl_2$-treated rats significantly improved the activities of these enzymes compared with only $HgCl_2$-treated rats ($p < 0.05$). There was no significant difference between the control and the only rutin 100 groups.

3.3. Rutin increases GSH content and decreases lipid peroxidation level

$HgCl_2$ administration leads to depletion of liver GSH content significantly as compared to control and only rutin 100 groups ($p < 0.05$). Treatment with rutin significantly increased GSH content at higher dose as compared to $HgCl_2$ treated group ($p < 0.05$).

$HgCl_2$ treatment evoked a significant increment in lipid peroxidation (LPO) as evidenced by increase in liver MDA level compared to the control and only rutin 100 groups. However, treatment with different concentrations of rutin significantly decreased the concentration of MDA when compared to $HgCl_2$-induced group ($p < 0.05$).

3.4. Anti-inflammatoric effect of Rutin on $HgCl_2$-induced inflammation

The evaluation for the anti-inflammatory effect of rutin was shown in Table 2. NF-κB, Bcl-3, IL-1β and TNF-α levels were increased in

Table 2
The effects of rutin on inflammatory parameters in $HgCl_2$-induced hepatotoxicity in rats.

Parameters	Control	Rutin 100	$HgCl_2$	$HgCl_2$ + Rutin 50	$HgCl_2$ + Rutin 100
NF-κB (ng/g tissue)	81.53 ± 0.76[a]	80.14 ± 0.94[a]	121.07 ± 1.60[d]	94.55 ± 1.02[c]	90.57 ± 0.71[b]
Bcl-3 (ng/g tissue)	10.83 ± 0.19[a]	10.95 ± 0.23[a]	17.34 ± 0.33[c]	15.12 ± 0.26[b]	14.75 ± 0.45[b]
TNF-α (ng/g tissue)	5.30 ± 0.07[a]	5.15 ± 0.04[a]	7.17 ± 0.06[d]	6.42 ± 0.05[c]	5.90 ± 0.05[b]
IL-1β (pg/g tissue)	42.67 ± 0.42[a]	41.58 ± 0.33[a]	57.42 ± 0.60[c]	47.22 ± 0.34[b]	46.27 ± 0.34[b]

Values are expressed as mean ± SEM of seven rats in each group. Different superscripts (a–d) in the same row indicate significant difference ($p < 0.05$) among groups. (NF-κB; nuclear factor kappa B, Bcl-3; B-cell lymphoma-3, TNF-α; tumor necrosis factor-α, IL-1β; interleukin-1β, p38α MAPK; p38α mitogen-activated protein kinase).

$HgCl_2$-treated rats as compared to normal control and only rutin 100 rats ($p < 0.05$). The rutin co-treatment (50 and 100 mg/kg b.w.) significantly decreased the elevation of NF-κB, Bcl-3, IL-1β and TNF-α levels in comparison with the $HgCl_2$-induced toxicity group ($p < 0.05$). There was no significant difference between the control and the only rutin 100 groups (Table 2).

3.5. Rutin decreases the p38α MAPK activitiy and p53 level

The treatment with $HgCl_2$ increased p38α MAPK activity and p53 level as compared to control and only rutin 100 groups ($p < 0.05$). However there were a marked decrease in p38α MAPK activities and p53 levels in case of groups treated with rutin dose dependently (Fig. 1A and B).

3.6. Rutin decreases $HgCl_2$-induced apoptosis

To study the molecular mechanisms of the anti-apoptotic effects of the rutin on $HgCl_2$-induced apoptosis in more detail, the expression levels of pro-apoptotic Bax, cytochrome c and anti-apoptotic Bcl-2 and procaspase-3 were investigated. Bax/Bcl-2 ratio was also analysed as it is more essential in determining apoptosis [37]. It was found that the Bax/Bcl-2 ratio was significantly increased by $HgCl_2$ and decreased by rutin (Fig. 2B-III). The level of cytochrome c increased significantly in the $HgCl_2$ treated group and significant decrease was observed in expression level of cytochrome c in the $HgCl_2$ + rutin 50 group (Fig. 2B-IV). Furthermore, the levels of procaspase-3 were significantly reduced and increased in the $HgCl_2$-induced and $HgCl_2$ + rutin 50 groups respectively (Fig. 2B-V). An increase in procaspase-3 level suggests a decrease in active caspase-3 level. These results further provide evidence that rutin could play an anti-apoptotic role in rats treated with $HgCl_2$.

3.7. Histopathological findings

The liver tissues of the rats in the control and only rutin 100 groups were observed to have a normal histological appearance. In the $HgCl_2$ group, there was hemorrhage in the liver parenchyma, and severe necrosis was observed in hepatocytes. Especially necrosis that started from the acinar region and moved towards the peripheral, severe hyperemia and serositis in the blood vessels were observed. In the $HgCl_2$ + rutin 50 group, especially in the hepatocytes in the acinar region, mild necrosis, medium-level hydropic degeneration and blood vessel hyperemia were determined. There was a statistically significant difference in comparison to the $HgCl_2$ group ($p < 0.05$). While there was mild degeneration in the hepatocytes of the liver parenchyma in the $HgCl_2$ + rutin 100 group, no necrosis was observed (Fig. 3). Histopathological findings were summarized in Table 3.

3.8. Immunohistochemical findings

EGFR expression in the liver tissues was examined immunohistochemically. EGFR expression was negative in the control and only rutin 100 groups. There were severe positivity values in the $HgCl_2$ group with a sinusoidal period, and EGFR expressions were on a severe level in the region where necrotic hepatocytes were abundant. EGFR expression in the hepatocytes in the central region in the $HgCl_2$ + rutin 50 group had moderate severity. There was a statistically significant difference in comparison to the $HgCl_2$ group ($p < 0.05$). There was mild EGFR expression in the central region in the $HgCl_2$ + rutin 100 group (Fig. 4). Immunohistochemical findings were summarized in Table 3.

4. Discussion

Inorganic mercury is an important environmental pollutant with adverse effects on multiple systems, though the specific mechanisms are not fully understood [7,38]. Inorganic mercuric ions, ingested in the

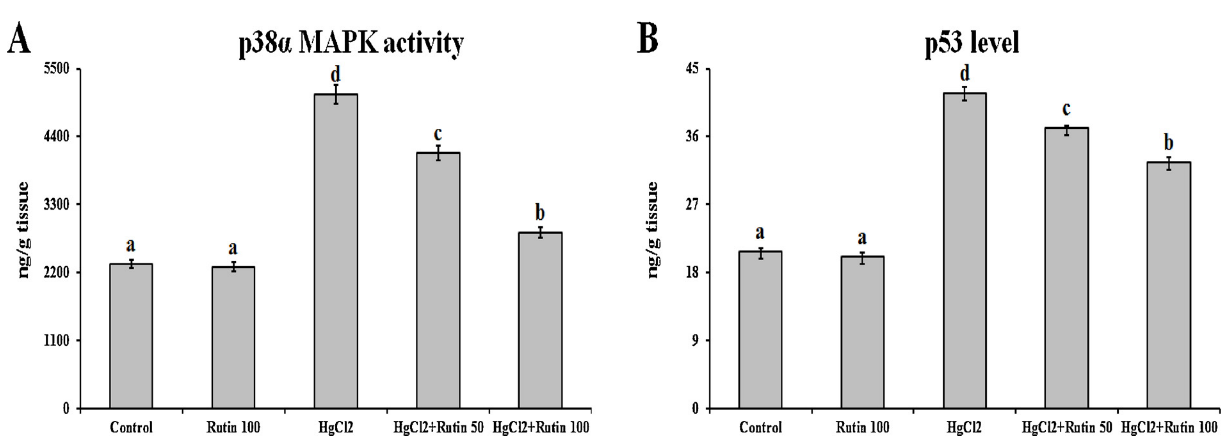

Fig. 1. (A) Effect of rutin treatment on $HgCl_2$-induced liver p38α MAPK in rats. (B) Effect of rutin treatment on $HgCl_2$-induced liver p53 level in rats. Data are expressed as means ± SEM. (a–d) different letters indicate statistical difference among the groups ($p < 0.05$).

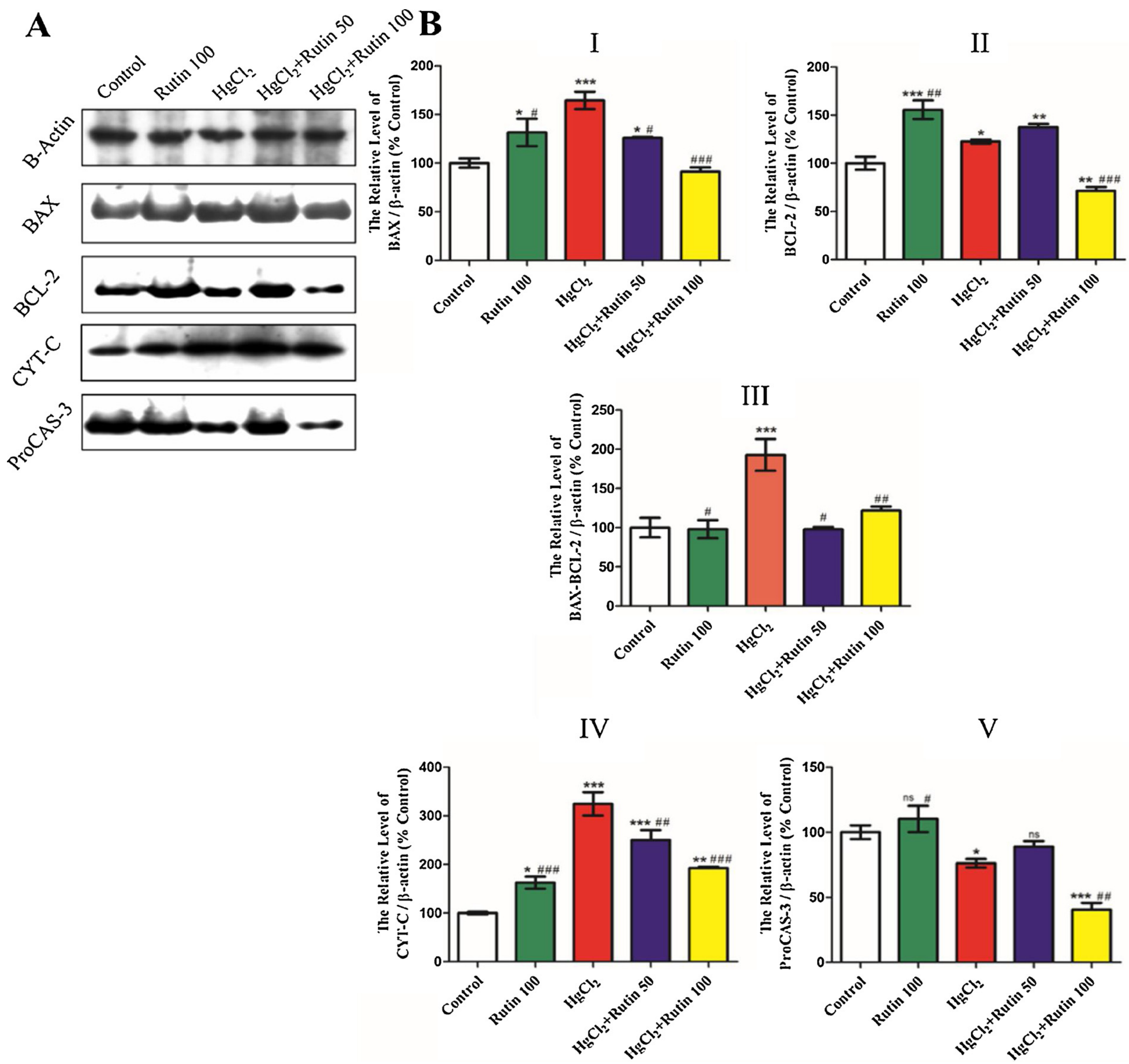

Fig. 2. Anti-apoptotic effects of rutin on $HgCl_2$-induced apoptosis. (A) Bax (23 kDa), Bcl-2 (26 kDa), Sitokrom *c* (15 kDa) ve procaspase-3 (34 kDa) protein levels were measured by Western blotting analysis. β-Actin was used as the loading control. (B) Data were shown as mean ± SEM. ***p < 0.001 Control vs Others, **p < 0.01 Control vs Others, *p < 0.05 Control vs Others; $^{###}$p < 0.001 $HgCl_2$ vs Others, $^{##}$p < 0.01 $HgCl_2$ vs Others, $^{#}$p < 0.05 $HgCl_2$ vs Others; ns: not significant.

diet, is poorly absorbed in the intestinal tract and most of it is excreted in the feces [39]. The absorbed inorganic mercury has been shown to have an extensive biliary-hepatic cycle. It is released into the bile and is partially reabsorbed into the circulatory system and then returned to the liver [39,40]. The accumulation of $HgCl_2$ in the liver induces oxidative stress and results in liver injury. Therefore, the aim of this study was to investigate ameliorative effects of rutin against $HgCl_2$-induced liver damage in rats.

The mechanism of hepatic injury induced by $HgCl_2$ is believed to involve free radical mediated damage and reactive oxygen species (ROS) production [7]. Inorganic mercury in the Hg^{2+} form is one of the most potent thiol binding agents for SH groups of endogenous biomolecules. Thus, it is connected to thiol-containing proteins and small-molecular weight thiols such as GSH and cysteine. In addition, mercuric ions can produce free radicals such as superoxide and hydrogen peroxide, which causes protein, lipid and DNA oxidation [9,39]. LPO is also thought to be a critical biomarker in the pathogenesis of $HgCl_2$-induced liver injury [40]. Increased level of LPO is resulted from the overgeneration of ROS and decreased activities of endogen antioxidant enzymes that lead tissue injury [9,41]. In various studies in support with our study, it has been well documented that $HgCl_2$ increases the generation of intracellular ROS levels and induces oxidative stress [7,28,40,42]. Previous studies have indicated that rutin has ameliorative effects against the liver injury induced by toxic agents such as cadmium and carbon tetrachloride [43,44]. In a similar study, it was also reported that rutin protects brain tissues against methylmercury-induced ROS and induces an increase in GSH levels [45]. Our data indicated that rutin attenuates free radical damage and oxidative stress, and enhances the antioxidant system including SOD, CAT, GPx and GSH, indicating that rutin provides protection against $HgCl_2$-induced oxidative stress and LPO.

Elevation of serum ALT, AST and ALP activities are important markers in the diagnosis of hepatocellular damage and liver diseases, as confirmed by Eldutar, Kandemir, Kucukler and Caglayan [46] and Abdel-Moneim, Dkhil and Al-Quraishy [47]. In present study, the administration of $HgCl_2$ results in significant increase in the levels of serum AST, ALT and ALP activities that is indicative of liver injury. This could be due to possible release of these enzymes from the cytoplasm, into the blood circulation straight after breaking the plasma membrane and cellular injury [9]. It has also been shown that inorganic mercury

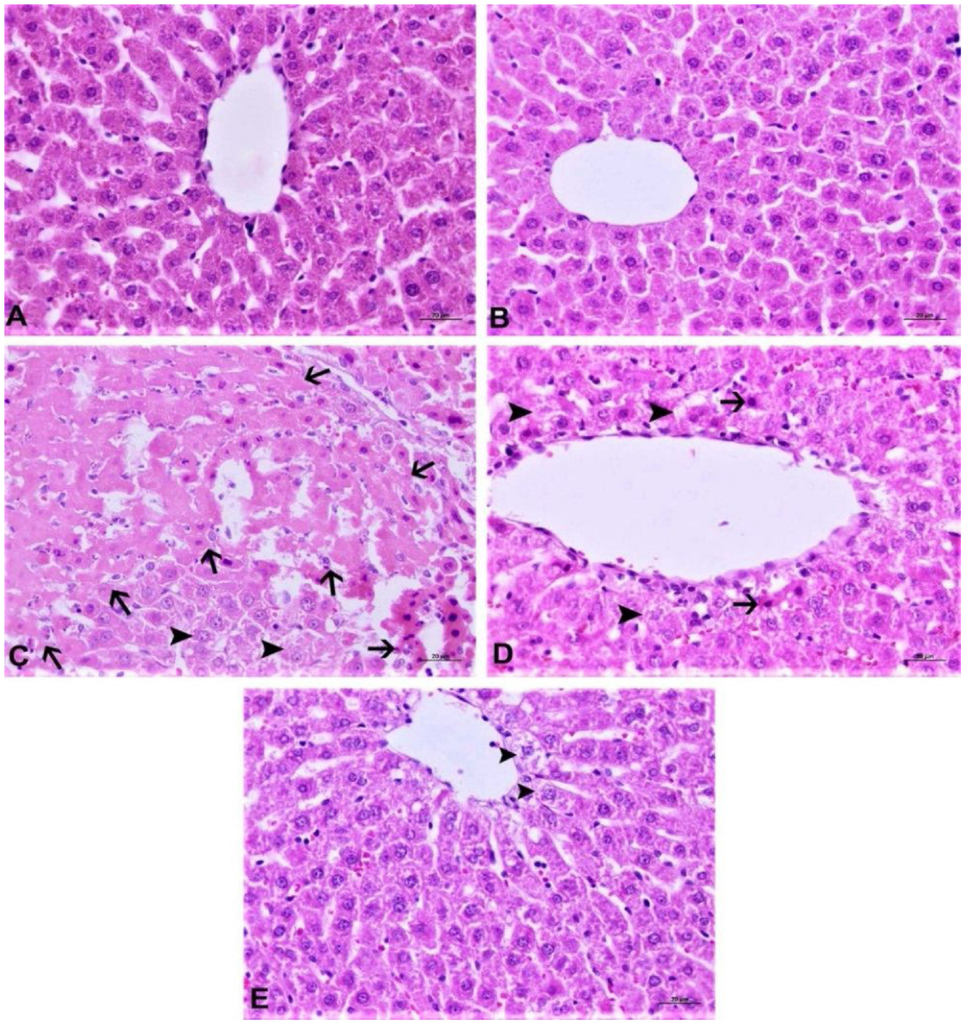

Fig. 3. Histopathological examination of rat liver tissue. (A and B) Control and rutin 100 group; normal histological appearance of liver tissue. (C) $HgCl_2$ group; severe necrosis (arrows) and degeneration (arrowheads) in hepatocytes. Also hyperemia and hemorrhage in the parenchymal tissue of liver. (D) $HgCl_2$ + rutin 50 group; moderate degeneration (arrowheads) and necrosis (arrows) were observed in hepatocytes in the acinar region. Also, sinusoidal hyperemia was observed. (E) $HgCl_2$ + rutin 100 group; hyperemia in the vessels and degeneration (arrowheads) in hepatocytes in the acinar region were seen in the vessels. H&E, Bar: 20 µm.

accumulation in liver tissues may be due to the deterioration of the enzyme system as a result of blockage of their active sites [48]. Histopathological examination showed that $HgCl_2$ caused destruction of the hepatic tissue which was supported by the degeneration, necrosis, hemorrhage and hyperemia in liver tissues. These biochemical and histopathological changes were prevented by rutin treatment in a dose-dependent manner. Rutin treatment was previously showed protective effects on liver morphology in carbon tetrachloride-induced hepatotoxicity in mice [49].

Inflammation is often linked with excessive ROS generation and oxidative stress and is thought to be important for $HgCl_2$-induced hepatotoxicity [2]. NF-κB is an important transcription factor known to be sensitive to oxidative stress, and plays a significant role in inflammation [50,51]. Under normal conditions, NF-κB interacts with inhibitory kappa B (IκB), remaining transcriptionally inactive [52]. Bcl-3 is an atypical member of the IκB family of NF-κB inhibitors. It is localized to the nucleus and acts as a co-activator of transcription factors participate in NF-κB signaling [53,54]. NF-κB and p53 could be upregulated by p38 MAPK. The p38 MAPK promotes the phosphorylation of IκB and results in the dissociation of NF-κB and IκB complexes [7,55]. Activation of NF-κB translocate into nucleus which then regulate the transcription of proinflammatory cytokine genes such as TNF-α, IL-1β and IL-6 [56–58]. In this study, administration of $HgCl_2$ significantly increased NF-κB, Bcl-3, TNF-α and IL-1β levels, and p38α MAPK activity reflecting inflammatory responses, whereas rutin showed anti-inflammatory effect by decreasing activation of these markers.

ROS is predominantly generated in mitochondria and plays a key role in apoptosis [59]. Apoptosis signaling pathways involves Bcl-2 and

Table 3
Histopathological and immunohistochemical finding and their scores in liver tissue in rats.

Parameters	Control	Rutin 100	$HgCl_2$	$HgCl_2$ + Rutin 50	$HgCl_2$ + Rutin 100
Necrosis in hepatocytes	–	–	+ + +	+	–
Degeneration of hepatocytes	–	–	+ + +	+ + +	+ +
Hyperaemia in the vessels	–	–	+ + +	+ +	+
EGFR expression	–	–	+ + +	+ +	+

(–) No change, (+) Mild change, (+ +) Moderate change, (+ + +) Severe change.

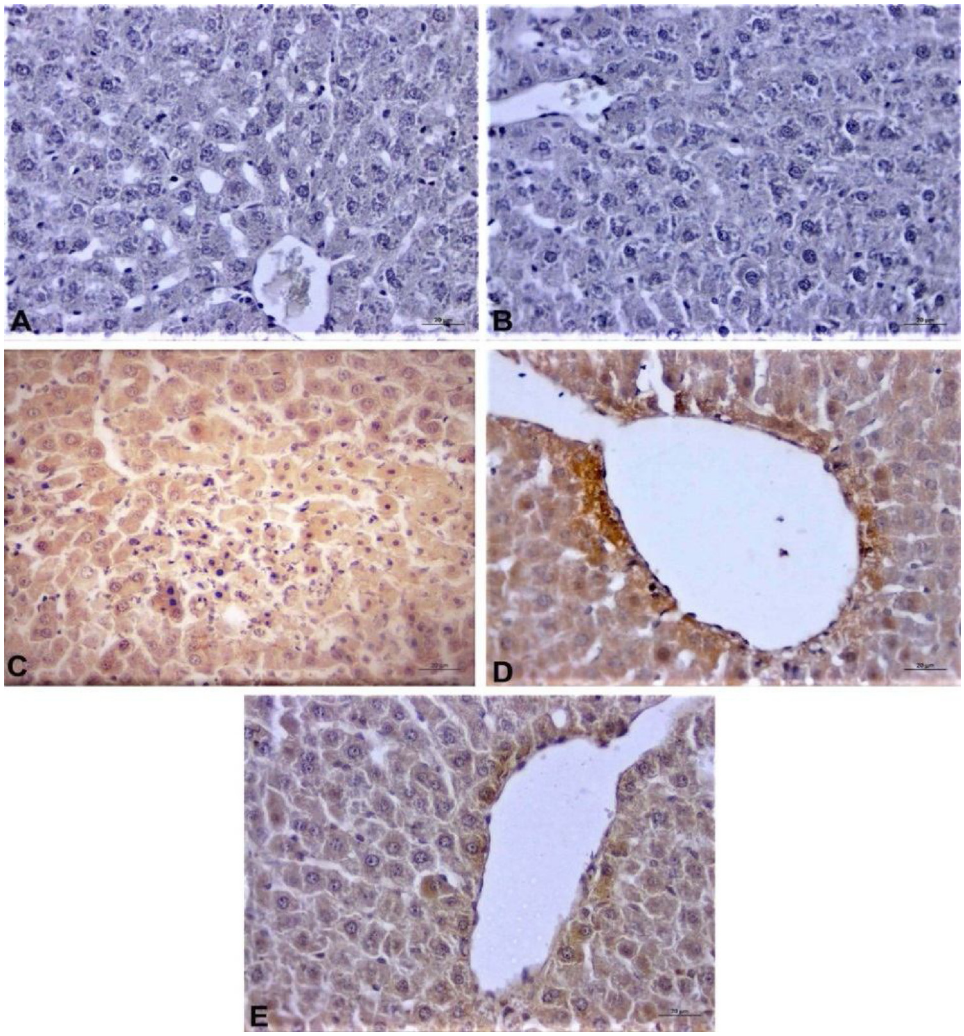

Fig. 4. EGFR expression in the rat liver tissue (A and B) Control and rutin 100 group; negative EGFR expression (C) $HgCl_2$ group; EGFR expression is severe positive around necrotic hepatocytes. (D) $HgCl_2$ + rutin 50 group; EGFR expression in the acinar region was moderate positive. (E) $HgCl_2$ + rutin 100 grup; EGFR expression in the acinar region was mildly positive. IHC-P, Bar: 20 µm.

the p53 protein family, including prosurvival and proapoptotic proteins. Tumor suppressor protein p53 affects apoptosis and may alter the levels of the Bcl-2 protein family [7]. Also, one of the important apoptotic pathways is the mitochondrial (intrinsic) pathway characterized by increased cytochrome c levels in the cytoplasm due to the impaired Bax/Bcl-2 balance. The increased level of cytochrome c promotes apoptosis, resulting in caspase-3 activation. The active caspase-3 is increased and thus induces apoptosis via intrinsic pathway [60–62]. It has been reported that mercury increases mitochondrial membrane permeability followed by the mitochondrial membrane potential reduction [57]. $HgCl_2$ was also reported to induce apoptotic pathways by increasing expression of Bax, caspase-3 and p53 in rat liver tissues [7]. Our immunoblotting studies demonstrated that $HgCl_2$ induced liver cell death through the over activation of p53, Bax, cytochrome c and caspase-3, and suppressed the expression of Bcl-2 protein level. However, rutin administered with $HgCl_2$ protects the cells by retaining the p53, cytochrome c, caspase-3, Bax and Bcl-2 expressions similar to control. In another study, the protective role of rutin in heart tissues against doxorubicin-induced apoptosis through inducing an increase in caspase-3 activity and decrease in Bcl-2 expression [63].

The epidermal growth factor receptor (EGFR), a plasma transmembrane glycoprotein, is expressed in various cells. When the ligand binds, EGFR activates its intrinsic tyrosine kinase activity [64]. The EGFR signalling pathway regulates cell differentiation, migration, proliferation, angiogenesis, apoptosis, protein secretion, and/or oncogenesis [64,65]. It has also been reported to play a key role during liver re-generation following acute and chronic liver injury, as well as in hepatocellular carcinoma and cirrhosis [66]. Activated EGFR is known to upregulate genes involved in inflammation like COX-2 expression through an EGFR-Ras-MAPKs-AP-1-COX-2 cascade [67]. Interestingly, COX-2-deficient mice showed an impaired liver re-generation [68]. However, there is no information on the relationship between EGFR expression and $HgCl_2$ exposure. The current study demonstrated that the $HgCl_2$-treated group stains more immunopositively for EGFR as compared to the control group, while dose-dependent rutin (50 and 100 mg/kg) treatments significantly decreased the EGFR immunopositive staining levels.

Although massive efforts are being sought in the search for new drugs against the toxicity of mercurial, there is no effective treatment that completely eliminates the toxic effects. In addition, chelating agents assist in reducing mercury body burden [45]. It has been also suggested that chelating therapies are ineffective in poisoning with organic forms of mercury such as methylmercury and ethyl mercury, although this issue remains controversial. Accordingly, the utility of compounds having antioxidant properties and having no apparent side effects may represent an effective adjuvant strategy to eliminate the toxicity caused by organic and inorganic mercury forms [69]. In this context, rutin has been reported to reduce specific oxidative damage markers induced by methylmercury in rat brain and induced by $HgCl_2$ in rat kidney tissues [10,70]. Future studies will in this area should further refine the mechanisms associated with the efficacy of rutin in reducing $HgCl_2$-induced toxicity, as well as assess the effectiveness of rutin in ameliorating systemic Hg-induced toxicity on clinical level.

5. Conclusion

In conclusion, the present study suggests that rutin has the potential to protect the liver from $HgCl_2$-induced damage. In addition, the ability of rutin to attenuate and reverse $HgCl_2$-induced oxidative damage, apoptosis, and inflammation in liver tissue can guarantee further in vivo studies in the search for potential antidotes to counteract $HgCl_2$-induced hepatotoxicity.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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