

Research Article

Protective Effect of DPPD on Mercury Chloride-Induced Hepatorenal Toxicity in Rats

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Mercury is a global environmental pollutant, accumulating mainly in the kidney and liver inducing hepatorenal toxicity, oxidative stress, and tissue damage. Oxidative stress is caused by an imbalance between free radicals' production and cellular antioxidant defense systems. In the present study, we investigated the effect of *N,N'*-diphenyl-1, 4-phenylenediamine (DPPD) antioxidant activity against mercury chloride- (HgCl_2 -) induced renal and hepatic toxicity. Thirty adult female Sprague Dawley rats were divided into three equal groups: the first group was injected with saline only and served as a control, the second group was injected with HgCl_2 , and the third group received DPPD + HgCl_2 rats injected with HgCl_2 without treatment showing a significant increase in alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, creatinine, and uric acids compared to control. Moreover, the second group showed a significant reduction in the activity of the antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH)) in addition to a marked increase in the malondialdehyde (MDA) content, histopathological alterations, collagen deposition, CD8%, CD4%, and TGF- β % in kidney and liver tissues compared with the control group. Treatment with DPPD showed significant recovery ($p \leq 0.001$) in all previous parameters and histopathological examination. In conclusion, we suggested that DPPD may have a promising antioxidant capacity, gives it the applicability to be used as a prophylactic agent against mercury-induced hepatorenal cytotoxicity in the future.

1. Introduction

Mercury is one of the most toxic metals responsible for environmental pollution [1]. Exposure to mercury in any of its forms in different ways such as water, air, soil, and food poses serious threats to our health and the environment [2]. Following exposure, mercury ions are taken up by and accumulate in numerous organs, including the brain, intestine, kidney, liver, and placenta [3]. Based on the available experimental data, it is a reasonable hypothesis that mercury toxicity involves oxidative stress, inflammation, and apoptosis [4]. HgCl_2 , as one of the most toxic salts of mercury, is

metabolized primarily in the liver and, then, accumulated in the kidneys. Consequently, the liver and kidneys are considered the most affected organs [5]. HgCl_2 demolishes free radical scavenging systems such as superoxide dismutase and catalase [6], as well as increase reactive species levels that lead to disturbance of the prooxidant-antioxidant balance system causing a condition of oxidative stress [7].

N,N'-diphenyl-1, 4-phenylenediamine, a grey or dark grey powder, is used as an antioxidant in rubber and oils, especially for tires in industry due to its colour and stability [8]. DPPD is one of the most frequently used and potent antioxidants. It is effective at very low concentrations and

believed to be rather selective [9]. DPPD, acting as an intracellular antioxidant, enlarges the pool size of lipid-soluble antioxidants, especially in the cytoplasmic membranes, and prevents the formation of lipid peroxides resulting in maintenance of the normal mitochondrial structure and enzyme activity [10]. The antioxidant activity of DPPD implemented by the donation of hydrogen to radical derivatives breaking the autocatalytic cycle protecting cells from oxidative stress [11] suppresses necrosis and decreases reactive oxygen species (ROS) formation [12]. Also, DPPD inhibits lipid peroxidation and nephrotoxicity [13]. Thus, DPPD inhibits collagen deposition, dampens apoptosis, and prevents histopathological damages [14]. The present study reports the antifibrotic effect of DPPD against hepatorenal fibrosis induced by HgCl₂ in rats.

2. Materials and Methods

2.1. Chemicals and Reagents. All chemicals and reagents were of the highest purity grade. DPPD ($\geq 99.8\%$) and HgCl₂ ($\geq 99.5\%$) were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). In addition to serum ALT, AST, and ALP activities, urea, uric acid, and creatinine levels were determined using colourimetric diagnostic kits (Biodiagnostic, Cairo, Egypt) according to the manufacturer's instructions. TGF- β %, CD4%, and CD8% were analyzed by using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). Data were quantified with C Flow software (BD Accuri, San Jose, CA). The hydroxyproline content is measured by ELISA as an important index reflecting the degree of kidney and liver fibrosis.

2.2. Animals and Experimental Design. Rats were assigned to groups by using the Statistical Package of Social Science (SPSS) program for Windows (Standard version 21). Thirty female Sprague Dawley rats, weighing approximately 170–220 gm, were purchased from the Medical Experimental Research Center (MERC), Faculty of Medicine, Mansoura University, Mansoura, Egypt. The animals were kept in polypropylene cages under standard laboratory conditions of relative humidity ($45 \pm 5\%$) and temperature ($25 \pm 2^\circ\text{C}$) with 12 h light/dark cycle and provided with food pellets and tap water ad libitum. Principles of laboratory animals caring (NIH publication no. 85–23, that revised 1985) were followed. Ethical protocols for laboratory animal care and use were approved and followed under the supervision of Faculty of Postgraduate Studies for Advanced Sciences (PSAS), Beni-Suef University, Experimental Animals Ethical Committee (No. BSU/EAEC/PSAS/16/112018).

Rats were randomly divided into 3 groups (10 rats/group). The HgCl₂ dose was 4 mg/kg, i.p.:

Group I (control): rats received saline i.p. for 14 days and served as the control

Group II (HgCl₂): rats were injected with a single dose of HgCl₂ (4 mg/kg, i.p.) at day one of the experiment

Group III (HgCl₂ + DPPD): rats were injected with a single dose of HgCl₂ (4 mg/kg, i.p.) at day one of the

experiments and, then, treated with DPPD (0.5 g/kg, i.p.) according to [15] once every two days starting from day 3 of the experiment

2.3. Collection and Preparation of Samples. All rats were exposed to sevoflurane anesthesia and killed by decapitation (24 h after the last injection), and urine and blood samples were collected from each rat after 14 days of HgCl₂ (or saline) injection. The liver and kidneys tissues were dissected and used for biochemical, flow cytometry, and histopathological examinations.

2.4. Liver and Renal Markers. To assess liver functions, serum ALT, AST, and ALP enzyme activities were detected. Also, serum urea, uric acid, and creatinine levels were determined to assess kidney functions using colourimetric diagnostic kits (Biodiagnostic, Cairo, Egypt) according to the manufacturer's instructions.

2.5. Oxidative Stress Evaluation. The activities of the antioxidant enzymes SOD, CAT, and GSH in addition to the MDA content in liver and kidney tissues were all measured using commercial laboratory diagnostic kits (Biodiagnostic Co., Cairo, Egypt).

2.6. CD8%, CD4%, and TGF- β % Measurements. Flow cytometry detection of CD8%, CD4%, and TGF- β % depends on the specific binding of monoclonal antibodies to the antigenic determinants. The monoclonal antibodies labelled with different fluorochromes which are excited via a laser beam from a flow cytometer during analysis. The fluorescence intensity differences were proportional to the expression of the analyzed antigens. Assays were analyzed by using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). Data were quantified with C Flow software (BD Accuri, San Jose, CA).

2.7. Measurement of the Hydroxyproline Content. About 50 mg of kidney or liver tissue specimens were hydrolyzed, and then, chloramine T solution was added to the specimen's supernatant and, then, incubated, followed by Ehrlich's solution addition. The final mixture was incubated, and the optical density was estimated at 560 nm [16]. Hydroxyproline values were expressed as $\mu\text{g}/\text{mg}$ tissue.

2.8. Histopathological Examination. The formalin-embedded liver and kidney tissues were cut into 4 μm thick sections, and then, the slides were stained with hematoxylin and eosin (H&E) for histological evaluation and Masson trichrome to assess collagen deposition. We routinely conduct H&E staining to grade tubular damage (0, no damage; 1, 0–25% damaged tubules; 2, 25–50% damaged tubules; 3, 50–75% damaged tubules; and 4, >75% damaged tubules) [17] and the liver injury score of fibrosis as described by [18]. The sections were examined and photographed using an Olympus light microscope (Olympus BX51, Tokyo, Japan)

TABLE 1: Kidney injury parameters. Values are expressed as $M \pm SD$ of 10 animals in each group.

Variables	Control group	HgCl ₂ group	HgCl ₂ + DPPD group
Serum:			
Creatinine (mg/dl)	0.46 ± 0.03	1.53 ± 0.21 ^a	0.57 ± 0.10 ^b
Urea (mg/dl)	27.83 ± 3.60	73.43 ± 5.87 ^a	32.23 ± 2.30 ^b
Uric acid (mg/dl)	2.57 ± 0.25	4.53 ± 0.76 ^a	3.16 ± 0.98 ^{a,b}
Homogenate:			
Hydroxyproline (ug/mg tissue)	22.58 ± 0.63	41.23 ± 9.25 ^a	30.24 ± 5.23 ^{a,b}
MDA (mmol/g tissue)	59.79 ± 4.99	92.25 ± 13.05 ^a	70.62 ± 9.69 ^b
SOD (U/mg protein)	11.66 ± 0.64	6.71 ± 1.51 ^a	9.63 ± 1.48 ^{a,b}
CAT (mol/min/gm)	0.66 ± 0.09	0.35 ± 0.05 ^a	0.58 ± 0.09 ^b
Glutathione (μmol/g protein)	32.13 ± 2.07	21.71 ± 6.22 ^a	28.21 ± 3.51 ^b
TGF-β (ng/ml)	33.27 ± 3.43	49.20 ± 9.10 ^a	38.60 ± 9.19 ^b
CD4 (ng/ml)	21.42 ± 1.04	43.70 ± 5.31 ^a	34.30 ± 5.01 ^{a,b}
CD8 (ng/ml)	23.62 ± 3.88	44.06 ± 6.77 ^a	25.66 ± 3.87 ^b

SD: standard deviation; P: probability; *significance <0.05; **high significance. The test used is one-way ANOVA followed by post hoc Tukey. ^aSignificance relative to the control group compared with HgCl₂ and HgCl₂ + DPPD groups. ^bSignificance between the HgCl₂ group and HgCl₂ + DPPD group.

with an attached digital photograph machine (Olympus E-330). Images were captured from each section randomly, and semiquantitative analysis of the fibrotic area was performed on an Intel® Core I5®-based computer using Image J software with a specific built-in routine for stain quantification and automated area measurement. Five slides were prepared from each group, 5 random fields from each slide analyzed as previously reported [19, 20].

2.9. Statistical Analyses. Data were analyzed using SPSS software version 22 for Windows (IBM, Armonk, NY, USA). Descriptive statistics were calculated in the form of Mean ± Standard deviation (SD). ANOVA and Tukey's post hoc tests were used for comparison between groups. A level of $p < 0.05$ was defined as statistically significant.

3. Results

3.1. Liver and Kidney Functions. The potential effects of HgCl₂ and DPPD treatment on renal and liver function parameters are summarized in Tables 1 and 2. The presented data showed that serum ALT, AST, ALP, creatinine, urea, and uric acid levels were significantly ($p < 0.001$) increased in HgCl₂-injected rats compared to control and HgCl₂ + DPPD-treated rats. Conversely, animals treated by HgCl₂ + DPPD reversed all parameters' alterations towards the normal ranges.

3.2. Lipid Peroxidation and Antioxidant Enzyme Activities. The data of lipid peroxidation, CAT, GSH, and SOD activities in the renal and hepatic tissues are shown in Tables 1 and 2. Compared with control and HgCl₂ + DPPD group values, the HgCl₂ group showed a significantly ($p < 0.001$) increased MDA level and significantly decreased antioxidant enzymes (CAT, GSH, and SOD) activities. These results indicate that DPPD ameliorates the HgCl₂-induced oxidative stress in the liver and kidney.

3.3. Hepatorenal Fibrosis Induced by HgCl₂ in Rats. The hydroxyproline content is a specific marker for collagen deposition. The HgCl₂ group showed a significantly ($p < 0.001$) increased hydroxyproline content in renal and liver tissues compared with the control group. In contrast, DPPD treatment significantly decreased the renal and liver hydroxyproline content (Tables 1 and 2).

3.4. Flow Cytometry. As shown in Tables 1 and 2, the TGF-β, CD4, and CD8 percent showed a significant ($p < 0.001$) increase in HgCl₂-treated rats compared to control and HgCl₂ + DPPD-treated rats. Conversely, animals treated by DPPD reversed TGF-β, CD4, and CD8 percent alterations towards the normal ranges.

3.5. Histopathological Analysis. H&E-stained and Masson trichrome-stained kidney sections are shown in Figure 1 and liver sections are shown in Figure 2. H&E histopathological stain in the control group showed normal kidney morphology (Figure 1(a)) and normal hepatic lobular architecture with distinct hepatocytes (Figure 2(a)). HgCl₂-treated animals showed tubular dilatation with many degenerated signs (Figure 1(b)) and hepatic degeneration with large areas of extensive cell necrosis (Figure 2(b)). Treatment with DPPD significantly attenuated the pathological changes in both kidney (Figure 1(c)) and liver (Figure 2(c)) tissues compared to the HgCl₂-treated group. Assessment of kidney (Figure 1(g)) and liver (Figure 2(g)) injury by a semi-quantitative scoring system from 0 to 5. Data were mean ± SD. * $p < 0.01$ vs. control, # $p < 0.01$ vs. HgCl₂.

The collagen content was assessed by Masson's trichrome stain, and the control group showed a normal collagen content in the kidney (Figure 1(d)) and liver (Figure 2(d)) tissues. Significant amounts of collagen deposition were observed in the kidney (Figure 1(e)) and liver (Figure 2(e)) tissues of HgCl₂-treated animals. Conversely, administration of DPPD + HgCl₂ showed a significant ($p < 0.001$) modulation in the collagen content level towards normal in both renal (Figure 1(f)) and hepatic (Figure 2(f))

TABLE 2: Liver injury parameters. Values are expressed as $M \pm SD$ of 10 animals in each group.

Variables	Control group	HgCl ₂ group	HgCl ₂ + DPPD group
Serum:			
ALT (U/L)	33.30 ± 3.30	64.70 ± 14.16 ^a	41.80 ± 11.14 ^b
AST (U/L)	59.40 ± 5.12	114.70 ± 15.96 ^a	74.00 ± 5.43 ^{a,b}
ALP (U/L)	215.45 ± 5.42	333.97 ± 32.48 ^a	247.10 ± 25.91 ^{a,b}
Homogenate:			
Hydroxyproline (ug/mg tissue)	19.80 ± 1.84	39.20 ± 1.77 ^a	24.36 ± 4.08 ^{a,b}
MDA (mmol/g tissue)	81.83 ± 4.58	112.72 ± 10.48 ^a	81.93 ± 4.02 ^b
SOD (U/mg protein)	20.99 ± 1.75	14.87 ± 2.65 ^a	19.94 ± 3.71 ^b
CAT (mol/min/gm)	1.05 ± 0.10	0.60 ± 0.28 ^a	0.93 ± 0.13 ^b
Glutathione (μmol/g protein)	25.35 ± 1.50	18.97 ± 3.10 ^a	22.24 ± 2.01 ^{a,b}
TGF-β (ng/ml)	32.31 ± 2.45	50.60 ± 8.83 ^a	34.60 ± 4.92 ^b
CD4 (ng/ml)	24.44 ± 2.64	39.66 ± 1.60 ^a	25.30 ± 3.31 ^b
CD8 (ng/ml)	19.73 ± 1.13	38.5 ± 2.88 ^a	21.07 ± 3.84 ^b

SD: standard deviation; P: probability; *significance <0.05; **high significance. The test used was one-way ANOVA followed by post hoc Tukey. ^aSignificance relative to the control group compared with HgCl₂ and HgCl₂ + DPPD groups. ^bSignificance between the HgCl₂ group and HgCl₂ + DPPD group.

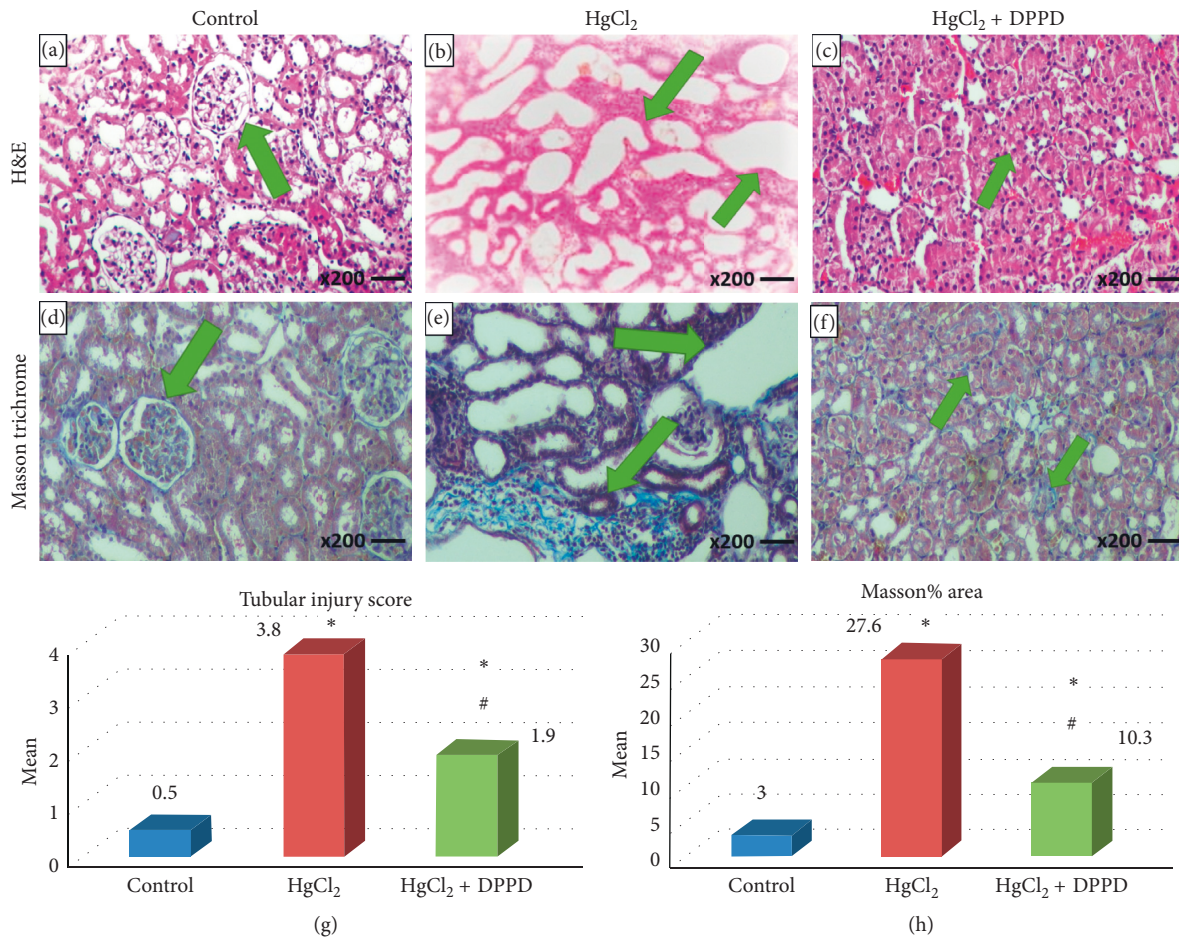


FIGURE 1: H&E-stained and Masson-stained kidney tissues of rats from different groups (magnification $\times 200$). (a) Section of the control group showing the normal architecture of the kidney. (b) Significant increase in tubular dilatation and degenerative changes observed in HgCl₂-injured rats. (c) Treatment with DPPD significantly attenuated the renal histopathological changes. (d) Masson's trichrome staining indicated no abnormal collagen in the control group. (e) Sections of HgCl₂-treated group indicated an increase in fibrosis stained in blue. (f) Kidney section of rats cotreated with HgCl₂ + DPPD showed a significant decrease in collagen deposits. (g) Pathological scoring showed a significant increase in the tubular injury score in the HgCl₂-treated group when compared with other groups. (h) Comparison between different groups in the Masson% area. Data were mean \pm SD. * $p < 0.01$ vs. control; # $p < 0.01$ vs. HgCl₂.

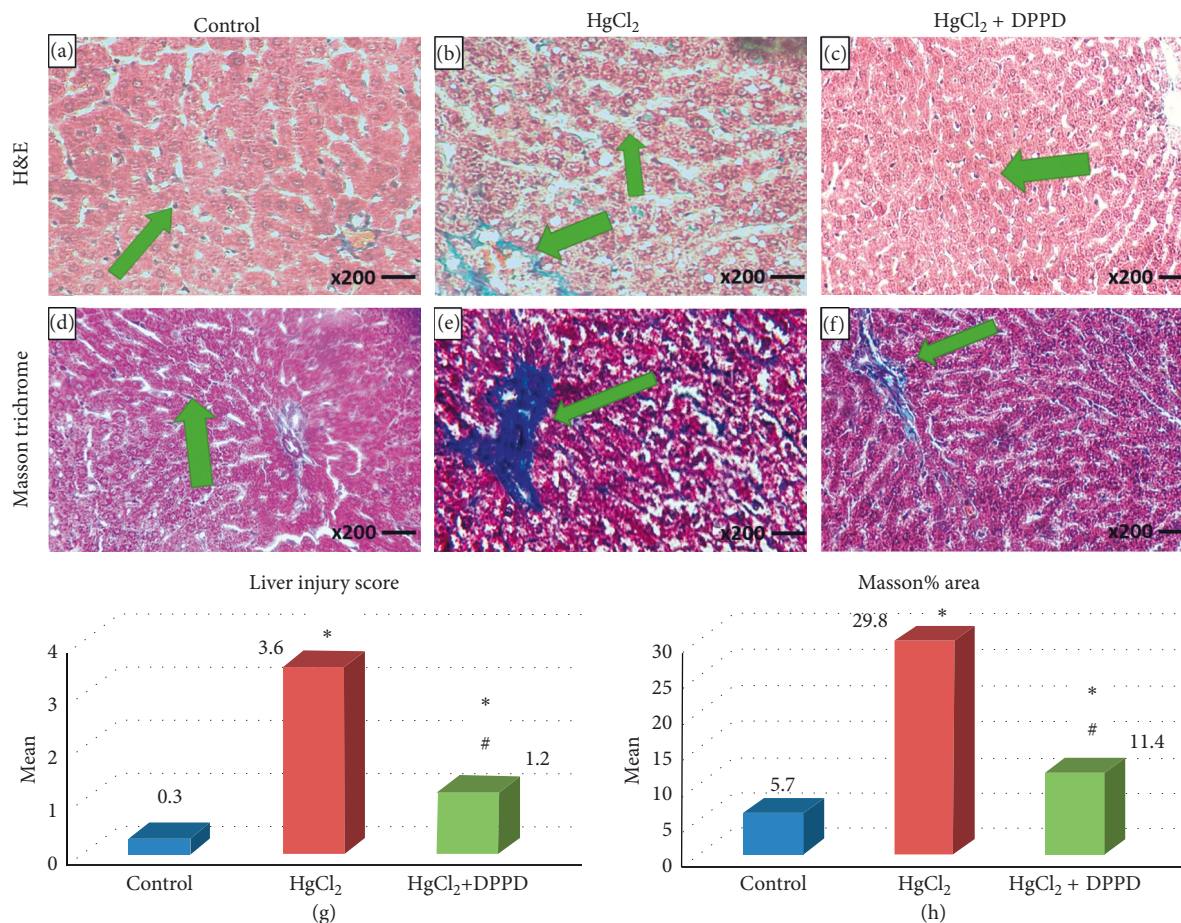


FIGURE 2: H&E-stained and Masson-stained liver tissues of rats from different groups (magnification $\times 200$). (a) Hepatic histology of the control group, showing normal hepatic lobular architecture. (b) Hepatic degenerative changes with extensive cell necrosis observed in HgCl₂-injured rats. (c) Rats treated with DPPD showed a significant modulation in the hepatic histology towards normal. (d) Control group stained with Masson's trichrome showed that the natural structure and collagen fibers cannot be seen. (e) Accumulation and progression of collagen fibers in the liver of the HgCl₂ group. (f) Significant decrease in collagen fibers observed in rats cotreated with HgCl₂ + DPPD. (g) Pathological scoring showed a significant increase in the hepatic injury score in the HgCl₂-treated group compared with other groups. (h) Comparison between different groups in the Masson% area. Data were mean \pm SD. * $p < 0.01$ vs. control; # $p < 0.01$ vs. HgCl₂.

tissues. Treatment with DPPD significantly ($p < 0.001$) decreased both the renal (Figure 1(h)) and hepatic (Figure 2(h)) Masson% area.

4. Discussion

HgCl₂ generates free radicals and subsequently increases oxidative stress, which leads to nephrotoxicity and accelerates hepatotoxicity [21]. This adverse effect of HgCl₂ could be eliminated by DPPD treatment probably because of its strong free radical scavenging activity through the electron(s) donation pathway, protecting cells from oxidation and necrosis [11].

In the present study, liver and renal functions were detrimentally altered after HgCl₂ administration causing hepatorenal dysfunction evidenced by a significant elevation in AST, ALT, and ALP enzyme activities and urea, uric acid, and creatinine levels. Similar results were reported by [22, 23]. Treatment with DPPD showed a marked improvement that was clear in the previously listed parameters,

and these findings agreed with those of Kawai et al. [13] who reported that DPPD may possess an antioxidative behavior.

HgCl₂ administration initiates the formation of highly reactive substances such as reactive oxygen species in addition to the stimulation of oxidative stress [24]. Consequently, the lipid peroxidation level increased and the antioxidant enzymes activities decreased.

In the present study, we found that HgCl₂ significantly diminishes the activities of the antioxidant enzymes SOD and CAT in addition to GSH in kidney and liver tissues, whereas the end product of lipid peroxidation (MDA contents) was significantly increased compared with the control group. A variety of experiments have demonstrated parallel results [23, 25]. Conversely, coadministration of DPPD + HgCl₂ showed a significant modulation in the activities of SOD and CAT in addition to the level of GSH and MDA towards normal. The hepatorenal protective activity of DPPD was observed in the previous studies [12, 26].

Hydroxyproline is used for the estimation of the collagen content, considering that collagen contained 12.7%

hydroxyproline by weight [27]. Our results were parallel to those of Yuan et al. [28] who concluded that renal and liver fibrosis are induced by HgCl₂, demonstrated by a significant elevation ($p \leq 0.001$) of the hydroxyproline content in liver and kidney tissues compared to control. Consequently, treatment with DPPD significantly attenuated hydroxyproline and collagen deposition ($p \leq 0.001$) in animals [19].

Our results reported a significant increase in both renal and hepatic TGF- β %, CD4%, and CD8% in HgCl₂-treated rats compared to (control and HgCl₂ + DPPD groups). The elevated CD4+ and CD8+ percentage may be related to the abnormal immune status due to HgCl₂ toxicity. These results are in agreement with the previous findings by Liu et al. [29], who indicate significant increases in CD4% and CD8% as a result of mercury induction of T-cell autoimmune syndrome, including autoantibodies and increases in TGF- β production and various other cytokines cause collagen deposition. Moreover, previous studies [30, 31] reported a significant increase in renal and hepatic TGF- β % in HgCl₂-treated rats compared to control, but rats treated with HgCl₂ + DPPD showed a significant decrease in TGF- β %, CD4%, and CD8% towards normal.

In the present study, we performed a histopathological examination to further support the biochemical and immunological evidence. We compared the morphological structure among each group using H&E stain. In the control group, there were no injuries or histological changes detected in the kidneys (Figure 1(a)) or liver (Figure 2(a)). The HgCl₂ group showed liver necrosis, swelling, and structure changes (Figure 2(b)) in addition to renal tissue damage, collagen formation, and atrophy in the normal tubular architecture (Figure 1(b)) compared with (control and HgCl₂ + DPPD groups). Treatment with DPPD significantly attenuated the pathological changes in both kidney (Figure 1(c)) and liver (Figure 2(c)) tissues. Earlier studies [4, 32] demonstrated parallel results.

Masson's trichrome stain was used for distinguishing collagen deposition. The control group showed a normal collagen content in the kidney (Figure 1(d)) and liver (Figure 2(d)) tissues, while in the HgCl₂ group, the collagen content was increased significantly in the kidney (Figure 1(e)) and liver (Figure 2(e)). Our results agreed with those in [33, 34]. The recorded hepatorenal lesions and collagen content in HgCl₂ + DPPD cotreated rats decreased significantly ($p < 0.001$) compared with HgCl₂-treated rats (Figure 1(f)) and (Figure 2(f)). Similar protective effects for DPPD were previously reported in [13].

5. Conclusions

Finally, we conclude that the antioxidant DPPD can retard the progression of hepatorenal fibrosis and collagen deposition induced by HgCl₂. Further studies are needed to explain the intrinsic and extrinsic pathways of DPPD antifibrotic efficacy.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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