

Research Article

Hemin Attenuates Cisplatin-Induced Acute Renal Injury in Male Rats

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Background. The aim of this study is to investigate the protective effects of hemin (the heme oxygenase-1 [HO-1] inducer) against nephrotoxic effects induced by cisplatin [cis-diamminedichloroplatinum II (CP)] in male rats. **Methods.** The evaluation was performed through monitoring renal redox parameters: lipid peroxidation (LPO), glutathione peroxidase (GPx), superoxide dismutase (SOD), glutathione reductase (GR), and reduced glutathione (GSH). The work also examined renal function tests (urea and creatinine), tissue proinflammatory mediator like nitric oxide (NO), and kidney cytopathology. **Results.** A single intraperitoneal dose of CP (10 mg/kg b.w.) caused significant elevation of blood urea, serum creatinine, and renal LPO and NO, along with significant decline of the activities of GPx and GR, but renal SOD activity and GSH level were statistically insignificant as compared to control group. Subcutaneous injection of hemin (40 μ mol/kg b.w.) partially ameliorated CP-induced renal damage, based on suppression of blood urea, serum creatinine, the renal MDA and NO levels, and increased antioxidant capacity in CP-treated rats. The results of histopathological and ultrastructural investigations supported the renoprotective effect of hemin against CP-induced acute toxicity. **Conclusion.** The induction of HO-1 by hemin is a promising approach in the treatment of CP-induced nephrotoxicity. However, further preclinical studies are warranted to test effectiveness of CP/hemin on the outcome of tumor chemotherapy.

1. Introduction

Cisplatin (cis-diamminedichloroplatinum(II), CP) is a highly effective chemotherapeutic agent against a large spectrum of tumor types [1–3]. However, the long-term clinical use of CP is limited by its serious side effects, mainly nephrotoxicity [4]. CP causes impairment of kidney function and acute renal failure via multiple mechanisms including generation of oxygen/nitrogen species, DNA damage, tubulointerstitial inflammation, and apoptotic cell death [5–12]. A number of studies have evaluated compounds as potential nephro-protectors against CP; these included natural antioxidants, modulators of nitric oxide synthesis, osmotic diuretics, and cytoprotective and antiapoptotic agents [13]. However, most

of them were not found suitable/safe for clinical practice. In this context, heme oxygenase-1 (HO-1), the rate-limiting enzyme in heme catabolism, might offer a promising alternative. HO-1 (also known as heat shock protein 32) is induced by free radical-initiated reactions, and its induction is considered to be an adaptive response against oxidative tissue damage [14–20]. In addition, HO-1 has been recognized to exhibit powerful anti-inflammatory and immunomodulatory effects [21]. Previous studies have shown that HO-1-inducing agents, as hemin, can mitigate nephrotoxic effects caused by a wide array of stressors, including mercury [15] and acetaminophen [20]. Based on the previous information, the present study aimed to examine whether the activation of HO-1 (by hemin) would have protective effects against CP

induced nephrotoxicity in rats. For this purpose, we have evaluated the status of renal lipid peroxidative assay and antioxidant defenses. In addition, detailed glomerular and tubular pathologies were assessed.

2. Materials and Methods

2.1. Drugs and Chemicals. CP and hemin (powder) were purchased from Sigma Chemical Company, USA. Other chemical reagents were of high-quality analytical grade. Hemin was first dissolved in 0.1 M NaOH, titrated to pH 7.4 with 0.1 M HCl, and then diluted with normal saline (1:10 v/v), while CP was prepared in normal saline.

2.2. Animals and Treatments. Healthy adult male rats (110–140 g) were obtained from animal house facility at King Saud University, Saudi Arabia. Rats were housed in polyethylene cages under controlled laboratory conditions and provided with standard rat chow and water *ad libitum*. They were allowed 1 week of acclimatization before the initiation of the experiment. Experimental protocol of this study complies with the NIH ethical guidelines for the manipulation and care of laboratory animals. Rats were randomly assigned into 4 groups ($n = 6$):

- (i) saline group (control): rats received 3 mL/kg 0.09% NaCl, intraperitoneally (i.p.);
- (ii) CP group: rats received a single dose of CP, i.p. (10 mg/kg);
- (iii) CP+hemin group: rats received 40 μ mol/kg hemin, subcutaneously (s.c.), 1 h following CP;
- (iv) hemin group: rats received 40 μ mol/kg hemin, s.c.

Doses, duration, and routes of exposure were chosen according to previously published reports [20, 22]. All rats were sacrificed under light ether anesthesia after 24 h of the last dose of specific treatment, and samples of trunk blood and kidneys were collected. Blood was centrifuged at 5000 rpm for 10 min and the separated sera were used for measurement of renal function tests. Kidneys were decapsulated and washed in cold isotonic saline. The cortex was carefully separated from medulla as described earlier by Banday et al. [23]. The kidney cortex was homogenized (Glass Col homogenizer) and a 20% w/v homogenate was prepared in ice cold 50 mM, pH 7.4 phosphate buffer saline. The homogenate was centrifuged at 5000 rpm for 20 min and the supernatant was then saved in aliquots to avoid sample thawing and freezing and stored at -80°C till used for assaying peroxidative damage and antioxidant status. Samples of the intact kidney tissues were used for light and electron microscopic studies.

2.3. Markers of Renal Toxicity. Serum levels of urea and creatinine were measured spectrophotometrically using commercial diagnostic kits (Human Gesellschaft für Biochemica und Diagnostica mbH, Germany), according to the methods described by Tabacco et al. [24] and Bartels and Böhmer [25], respectively.

2.4. Oxidative Stress-Related Indices. Malondialdehyde (MDA), an index of fatty acid oxidation, was estimated in quantifiable amounts using Thiobarbituric Acid Reactive Substances (TBARS) assay kit (BioAssay Systems, CA, USA) according to the method of Ohkawa et al. [26]. In this procedure, MDA reacts with thiobarbituric acid (TBA) to form a pink-colored complex that has maximum absorbance at 532 nm. MDA value was calculated in terms of nmol/g wet tissue. Nitric oxide (NO) level was determined in kidney homogenates using Nitrate/Nitrite Colorimetric Assay Kit (BioAssay Systems, CA, USA) according to the manufacturer's instructions. NO production was measured following reduction of nitrate to nitrite using improved Griess method [27]. Total NO synthetase (NOS) activity was detected by NOS assay kit (BioAssay Systems, CA, USA) [28]. Activities of glutathione peroxidase (GPx, EC 1.11.1.9) and superoxide dismutase (SOD, EC 1.15.1.1) as well as the level of reduced glutathione (GSH) in renal cortex were determined spectrophotometrically, according to the standard detection protocol of analysis kits (BioAssay Systems, CA, USA) [29–31]. Glutathione reductase (GR, EC 1.6.4.2) activity was assayed using a commercial kit from Cayman Chemical Company, USA [32]. Protein content was estimated by the method of Lowry et al. [33].

2.5. Light Microscopy. Kidney taken from each animal was fixed in 10% formalin solution, dehydrated in ascending series of ethanol, and embedded in paraffin. Sections (4 μ m-thick) were cut, stained with haematoxylin and eosin solutions, and examined under light microscope (Nikon 80i, Japan).

2.6. Electron Microscopy. Small slices of kidney cortex ($n = 3$ per group) were fixed in 3% glutaraldehyde in sodium phosphate buffer (200 mM, pH 7.2) for 3 h at 4°C . Postfixation was in cold 1% osmium tetroxide (Agar Sci. Ltd., England) for 1 h. After flushing in phosphate buffer, the tissue samples were dehydrated in graded ethanol solutions and embedded in Araldite (Agar Sci. Ltd., England). Ultrathin sectioning (80–100 nm) was carried out using Leica EM UC6 (Leica Co., Austria) ultramicrotome. Sections were mounted on grids, double stained with 2% uranyl acetate and lead citrate, and viewed under Jeol JEM 1011 transmission electron microscope (Jeol Ltd., Japan) at 80 kV.

2.7. Statistics. All variables were compared using one-way analysis of variance (ANOVA) followed by LSD multiple range test. Differences at $P < 0.05$ were considered significant. Statistical tests were performed using SAS statistical software (SAS v. 9.2, SAS Institute, Inc., Cary, NC). Data were presented as mean \pm standard error (SE).

3. Results

3.1. Biochemical Findings. The results of biochemical analysis in all studied groups are shown in Table 1. Treatment of male rats with CP resulted in significant increases in levels of blood urea (3.1-fold) and creatinine (6.3-fold) compared to control animals, indicating renal damage. These changes were

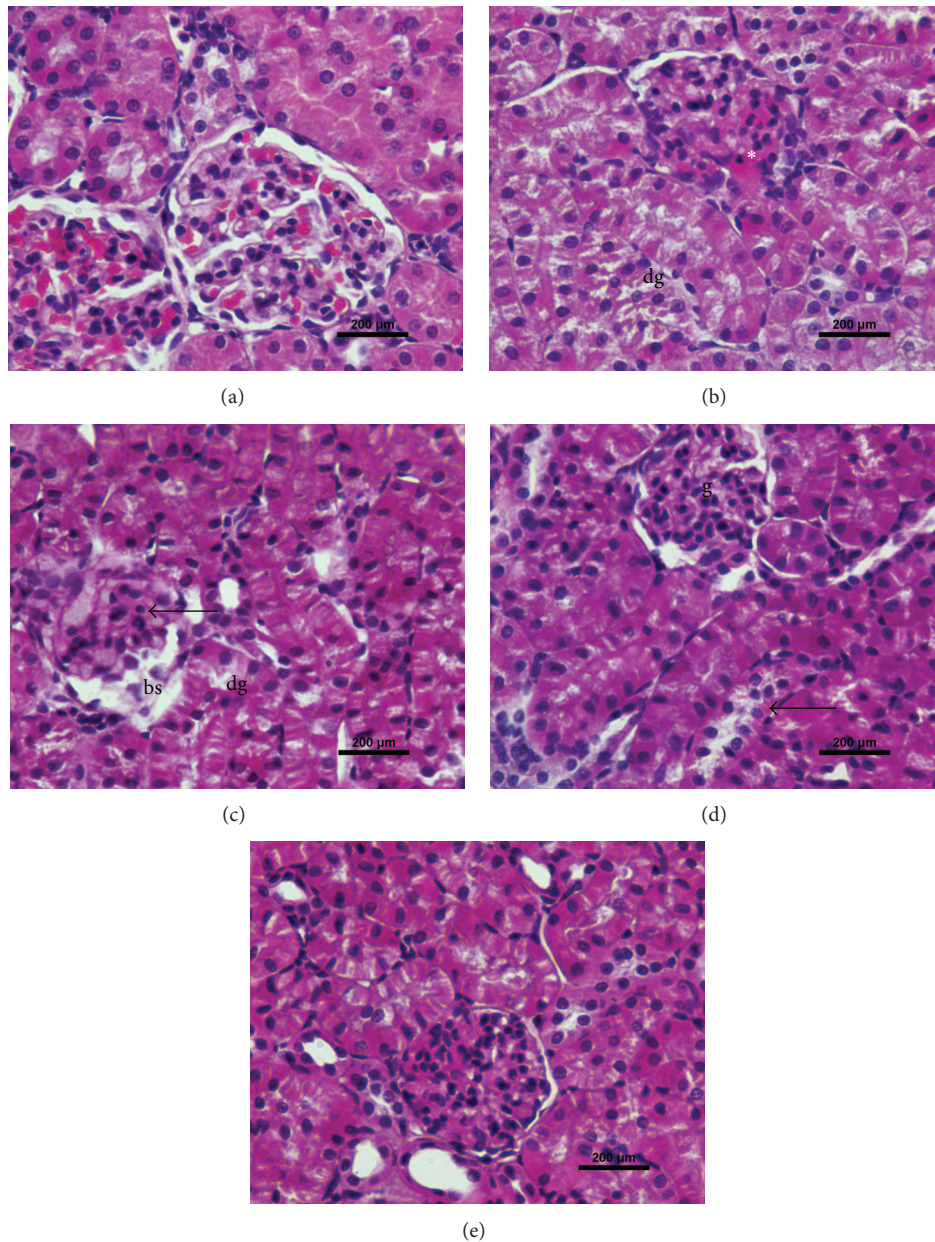


FIGURE 1: Photomicrographs of rat kidney. (a) Control group: normal histological structure. ((b)-(c)) CP group: severe glomerular congestion (*, in (b)), glomerular atrophy (arrow, in (c)) and dilatation of Bowman's space (bs, in (c)), and degeneration of renal tubular cells (dg, in (b) and (c)). (d) CP plus hemin group: mild tubular degeneration (arrow), a normal-looking glomerulus (g) is also discernible. (e) Hemin group: renal histology is comparable to control.

significantly reversed by hemin treatment. Hemin administration alone had no effect on kidney function. With respect to TBARS, renal levels were significantly higher in CP group than the control values, while TBARS levels were similar in CP plus hemin and control groups. The levels of NO in cortical tissue were significantly increased in rats injected with a single dose of CP as compared to control animals. Hemin treatment significantly decreased the elevated renal NO levels. In a similar manner, an elevation in NOS activity (4.6-fold) was observed in CP group compared to control group. In rats treated with hemin plus CP, the activity of NOS was decreased by 36.2% compared to CP-treated animals. On the

other hand, the activities of GPx and GR were profoundly declined by approximately 30% in cortical homogenates of CP group compared to the corresponding control values. Administration of hemin prevented CP elicited decreases in activities of these antioxidant enzymes. On the contrary, no statistically significant changes in SOD activity and GSH level were recorded after CP treatment; however, hemin administration either alone or in combination of CP enhanced renal GSH levels compared to the control group.

3.2. Kidney Histopathology. Representative light micrographs of renal cortex are illustrated in Figure 1. The histological

TABLE 1: The results of biochemical analysis in control and experimental groups^a.

Parameters	Unit	Control	CP	CP + hemin	Hemin
Urea	mg/dL	48.60 ± 1.50	151.61 ± 10.02 ^b	86.61 ± 3.02 ^{b,c}	53.60 ± 3.20 ^c
Creatinine	mg/dL	0.81 ± 0.11	5.14 ± 0.76 ^b	1.45 ± 0.69 ^c	0.78 ± 0.08 ^c
TBARS	nmole MDA/g tissue	6.44 ± 1.67	11.00 ± 1.05 ^b	7.26 ± 2.74	4.06 ± 1.53 ^c
NO	μmole/g tissue	16.42 ± 0.98	19.58 ± 1.50 ^b	13.16 ± 0.71 ^{b,c}	11.86 ± 0.84 ^{b,c}
NOS	nmole/g tissue	5.56 ± 0.84	25.61 ± 7.50 ^b	16.34 ± 2.19	6.07 ± 0.89 ^c
GPx	mU/mg protein	353.51 ± 44.43	238.49 ± 40.83 ^b	311.15 ± 25.76	376.81 ± 30.18 ^c
SOD	U/mg protein	18.14 ± 2.76	14.01 ± 1.85	17.59 ± 1.83	18.08 ± 1.15
GR	mU/mg protein	98.79 ± 8.40	67.63 ± 6.03 ^b	86.26 ± 9.13	105.75 ± 10.73 ^c
GSH	μmole/mg protein	6.68 ± 1.10	6.93 ± 0.59	9.15 ± 1.01 ^b	10.05 ± 0.37 ^{b,c}

TBARS: thiobarbituric acid reactive substances, MDA: malondialdehyde, NO: nitric oxide, NOS: nitric oxide synthetase, GPx: glutathione peroxidase, SOD: superoxide dismutase, GR: Glutathione reductase, and GSH: reduced glutathione.

^aResults are expressed as mean ± SE for six replicates.

^bSignificantly different from control at $P < 0.05$ by one-way ANOVA.

^cSignificantly different from CP at $P < 0.05$ by one-way ANOVA.

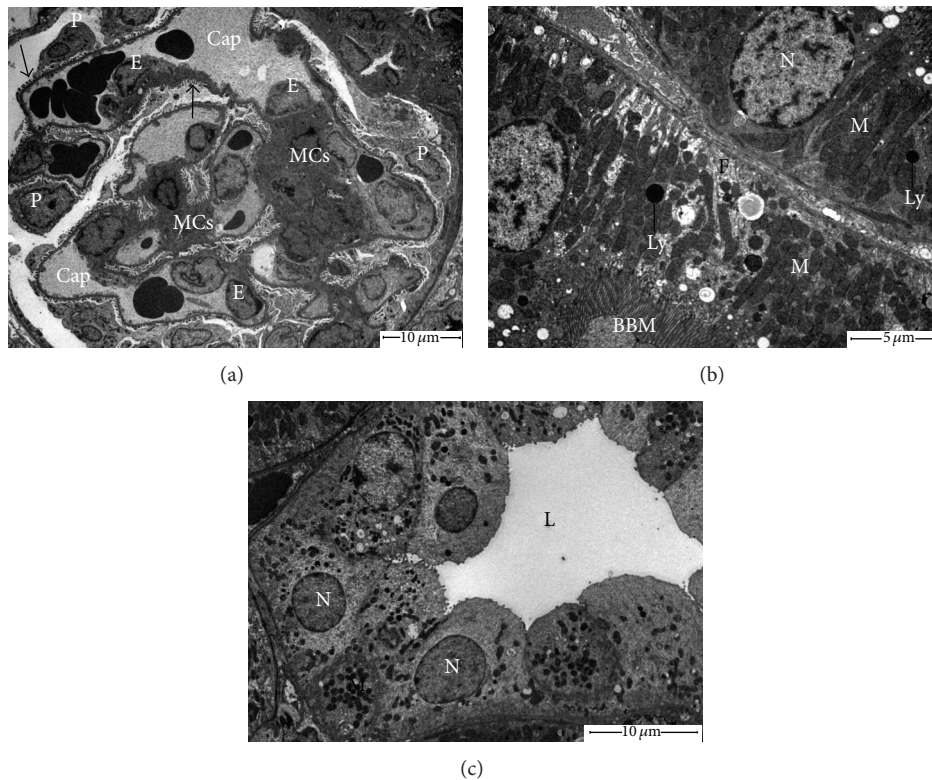


FIGURE 2: Electron micrographs of kidney of control rats. (a) Note the glomerulus has podocytes (P) which remain in contact with GBM by foot processes (arrows). The glomerular capillaries (Cap) are lined by endothelial cells (E) which are richly fenestrated and supported by mesangial cells (MCs) with their surrounding extracellular matrix. (b) Proximal tubules have well-developed brush border microvilli (BBM), and their lining epithelia contain large number of mitochondria (with normal feature) (M), few lysosomes (Ly), and many basal infoldings (F), N: nucleus. (c) Epithelial lining of distal tubule with apical nuclei (N), numerous mitochondria (M), short cisternae of rough endoplasmic reticulum, and few microvilli toward the lumen (L).

picture of kidney was normal in both control (Figure 1(a)) and hemin-treated (Figure 1(e)) groups. In CP-treated rats (Figures 1(b)-1(c)), the renal corpuscles displayed extensive congestion filling up the glomerular capillary loops. Some glomeruli were atrophied or lost with concurrent dilatation of Bowman's space. The morphological deterioration was also

characterized by a widespread tubular cell swelling, necrosis, and degeneration, occurring primarily in proximal convoluted tubules (PCTs) epithelia. In addition, peritubular inflammatory cell infiltrations and hemorrhagic foci were clearly apparent after CP treatment. In contrast, these histological abnormalities were found to be reduced in CP plus

TABLE 2: Semiquantitative scoring of glomerular and tubulointerstitial lesions in control and experimental rats.

Group	Control	CP	CP + hemin	Hemin
Glomerular congestion	—	++	+	—
Glomerular atrophy	—	++	+	—
Peritubular inflammatory cell infiltration	—	+++	+	—
Tubular damage	—	+++	+	—

Scoring scale: none (—), mild (+), moderate (++), and severe (+++).

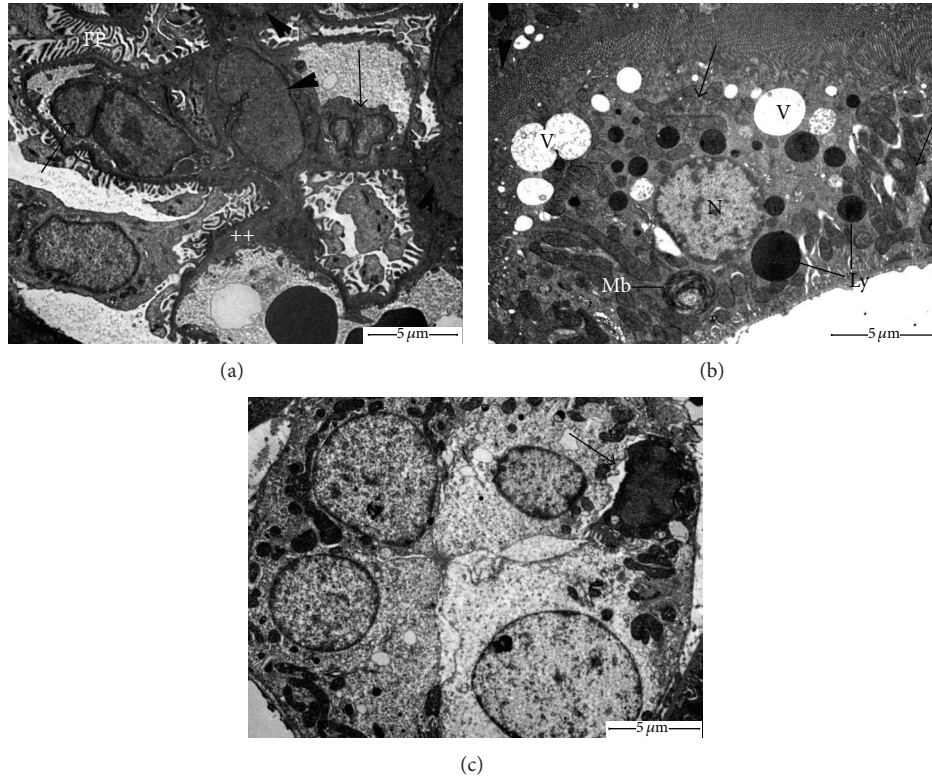


FIGURE 3: Electron micrographs of kidney of CP-treated rats. (a) Renal glomerulus with thickened GBM (++) , capillary lumens are obliterated by endocapillary hypercellularity and hypertrophy (arrows). Note also fusion of secondary foot processes (FP) and mesangial hypercellularity (arrowheads). (b) Proximal convoluted tubule cell containing large cytoplasmic vacuoles (V), clusters of deformed mitochondria (arrows), numerous lysosomal bodies (Ly), and lamellar (myeloid) body (Mb). Observe nucleus (N) with condensed chromatin pattern. Arrowhead points to partial destruction of the brush border. (c) Abnormal distal tubular cells with few organelles. Arrow indicates apoptotic cell.

hemin-treated rats (Figure 1(d)). Table 2 shows a semiquantitative analysis of renal lesions in rats treated with CP with or without hemin.

3.3. Electron Microscopic Observations. Renal cortical tissues of control animals revealed normal appearance of ultrastructural patterns of renal corpuscles and tubules (Figures 2(a)–2(c)). Obvious ultrastructural changes were noted in the glomeruli of CP-treated rats (Figure 3(a)). We observed progressive deformation of capillary endothelial cells, including hypertrophy (i.e., swelling), hyperplasia, and focal loss of fenestrae. The secondary foot processes of podocytes were deteriorated and markedly fused in some places. The Glomerular Basement Membrane (GBM) was corrugated and displayed uneven thickening. The mesangium was expanded with massive increase in matrix. Examination of PCTs of CP group

revealed the presence of destructed/irregular microvilli, apical vacuoles, large secondary lysosomes, altered mitochondria, and accumulation of myeloid bodies in the cytoplasm (Figure 3(b)). The distal convoluted tubules (DCTs) epithelia of CP-treated rats lost most of their cytoplasmic electron density and cellular organelles (Figure 3(c)). Apoptotic cells were also seen and identified by cell shrinkage, degradation of chromatin, and membrane blebs. CP plus hemin group showed marked recovery in the ultrastructural aspect of glomeruli and cellular features of renal tubules (Figures 4(a)–4(c)).

4. Discussion

Kidneys are dynamic organs and represent one of the major homeostasis body systems; they are affected by diverse varieties of chemicals and drugs [34–36]. Platinum complexes

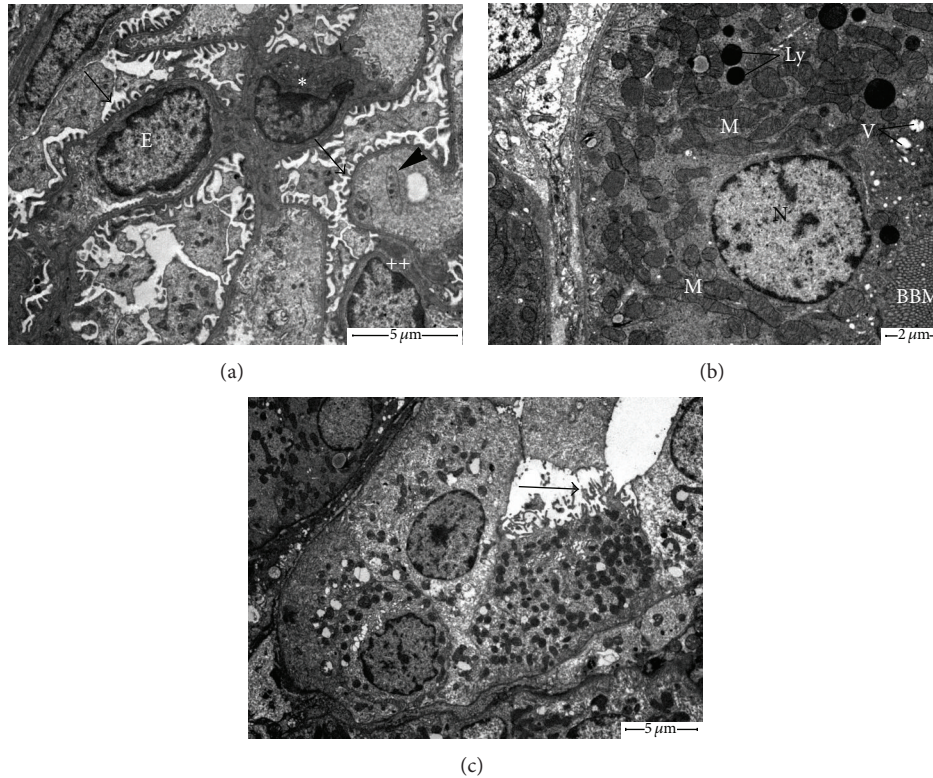


FIGURE 4: Electron micrographs of kidney of CP plus hemin-treated rats. (a) Glomerulus with nearly regular foot processes (arrows) and limited mesangial matrix (*). Arrowhead indicates a resting platelet inside glomerular capillary vessel. Capillary endothelium (E) and slight thickening of GBM (++) are indicated. (b) Proximal convoluted tubule cell showing loss of large vacuolar structures; only small vacuoles (V) are seen beneath the intact brush border microvilli (BBM). Nucleus (N), mitochondria (M), and lysosomes are of normal morphology. (c) Dilated distal tubule with normal cellular ultrastructure. Detachment/loss of pleomorphic microvilli is indicated by arrow.

(such as CP) are highly nephrotoxic, which predominantly accumulate in the kidneys [37]. It is evident from the present study that administration of a single dose of CP (7.5 mg/kg i.p.) resulted in acute tubule cell necrosis, interstitial inflammation, and glomerular congestion and atrophy. Besides, renal function biochemical parameters such as blood urea and creatinine were markedly elevated in CP-treated rats compared to control group, reflecting early damage of the filtration barrier (i.e., the fenestrated capillary endothelium, GBM, and visceral epithelial podocytes). These results are in agreement with previous reports in murine models [37, 38]. Hemin significantly suppressed the increases in blood urea and serum creatinine, which may be due to improvement in glomerular filtration damage induced by CP.

Development of therapies to prevent free radicals generation may influence renal oxidative damage and protect against irreversible cell damage and necrosis induced by CP. Excessive ROS production by CP causes antioxidant imbalance and leads to progressive LPO and antioxidant depletion. LPO adducts were previously shown to mediate glomerular lesions [39]. In addition, peroxidation of membrane phospholipids increases membrane fluidity and permeability and results in hypertrophy (i.e., swelling) of renal tubule cells and degeneration of cell organelles, such as nuclei and mitochondria [40]. ROS production is further stimulated by damaged mitochondria in CP nephrotoxicity [8]. Our results

showed that CP induced a significant elevation in MDA (a secondary product of LPO) and decrease in activities of GPx and GR, which are in agreement with previous studies [11, 41–44]. In line with these data, the formation of large myeloid bodies (i.e., multilamellated lysosomes), as observed in PCTs epithelia of CP group, has been a hallmark alteration indicative of an inhibited or altered function of intracytoplasmic enzymatic machinery [45]. The administration of hemin decreased or abolished kidney oxidative stress/LPO and increased antioxidant potentials. Hemin was reported to induce HO-1 protein which exerts cellular protection against renal toxicants [15]. HO-1 expression blocks injury pathways by catalyzing the breakdown of prooxidant heme into biliverdin (BV), carbon monoxide (CO), and ferrous iron (Fe^{+2})/ferritin. BV formed in this reaction is rapidly recycled by the action of biliverdin reductase into bilirubin (BR) which has a higher free radical-scavenging activity and antiapoptotic properties [46–49]. In addition, CO triggers the nuclear factor-erythroid 2-related factor 2 (Nrf2) to increase the expression and function of a battery of antioxidant enzymes [50]. Kim et al. [51] demonstrated that hemin substantially stimulates nuclear translocation of Nrf2 and its subsequent binding to antioxidant responsive element (ARE), a regulatory enhancer sequence in the promoter region of the involved genes.

Treatment with CP overdose induces nitrosative stress by NO and other nitrosylating agents and this was correlated with the expression of the inducible nitric oxide synthase (iNOS) protein [52]. In the current study, NO production in the CP-treated group was significantly higher than that in the control group; our results are consistent with the results obtained by Kart et al. [53], who have shown that there is strong immunoreactivity against iNOS in the liver tissue of the CP-treated group. Recent work of Chirino et al. [54] reported that the downregulation of iNOS expression reduced CP-induced renal damage and nitrosative stress. High NO levels exert toxicological effects by reacting with superoxide anion to generate short-lived but hyperactive peroxynitrite radical with subsequent nitration of protein tyrosine residues [55, 56]. Also, NO output depletes intracellular GSH, which increases susceptibility to oxidative stress and aggravates renal tissue damage [57, 58], especially for glomerular diseases (e.g., lupus nephritis) [59]. Hemin-mediated augmentation of HO-1 activity was proved to be efficient enough to reduce the NO-dependent pathological and inflammatory conditions [17, 60, 61]. Of note, HO-1 and its reaction product CO also suppress the expression of iNOS protein by preventing the activation of nuclear factor-kappa B (NF- κ B) which upregulates the transcription of the iNOS gene [17, 62, 63].

In conclusion, this study is the first to demonstrate the protective role of hemin (an HO-1 activator) against acute nephrotoxicity induced by CP. Treatment of hemin ameliorated renal ultrastructural changes and dysfunction, and this was associated with reduction of LPO, overstimulation of antioxidant capacity, and suppression of NO biosynthesis, the proinflammatory mediator. However, further preclinical studies are needed to verify whether coadministration of CP and hemin could affect the outcome of tumor chemotherapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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