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# Clemizole hydrochloride, a potent TRPC5 calcium channel inhibitor, prevents cisplatin‐induced nephrotoxicity in Spraque–Dawley rats

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## Abstract

Cis‐diamminedichloroplatinum (II) (cisplatin, Cis) is widely employed to treat several types of cancer. It has many important toxic side effects; one of the most important of which is nephrotoxicity. Clemizole hydrochloride (Clem) as the most potent inhibitor of TRPC5 channels was tested in an animal model of Cis‐induced nephrotoxicity. Rats were divided into the following groups: control; Cis (8 mg/kg); Cis + 1 mg/kg Clem; Cis + 5 mg/kg Clem; Cis + 10 mg/kg Clem. Kidney injury was detected by histopathological and biochemical analysis. Urine urea nitrogen (UUN), creatinine, urine neutrophil gelatinase‐associated lipocalin (NGAL), serum catalase (CAT), and malondialdehyde (MDA) levels were determined by enzyme‐linked immunosorbent assay. Total antioxidant status (TAS) and total oxidant status (TOS) were studied using a colorimetric assay. Nephrin, synaptopodin, and Rac family small GTPase 1 (RAC1) expressions were detected by Western blot analysis. Cis was found to induce histopathological alterations, including tubular degeneration, congestion, hemorrhage, hyaline casts, glomerular collapse, and apoptotic cell death. Clem at a dose of 1 and 5 mg/kg attenuated histopathological alterations. UUN, creatinine, and NGAL levels increased in the Cis‐administered group, while all doses of Clem decreased in those. CAT and TAS levels decreased, while TOS and oxidative stress index levels increased in the Cis‐treated group. A dose of 1 and 5 mg Clem showed antioxidant effects against oxidative stress. Cis induced lipid peroxidation by increasing MDA levels. All doses of Clem reduced MDA levels. Nephrin and synaptopodin expressions were decreased by Cis, and all doses of Clem increased that. All doses of Clem successfully depressed RAC1 expression. Clem showed a highly ameliorating effect on toxicity caused by Cis by blocking TRPC5 calcium channels.

**KEYWORDS**

cisplatin, clemizole, nephrotoxicity, TRPC5

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## 1 | INTRODUCTION

Cis-diamminedichloroplatinum (II) (cisplatin, Cis) is a drug widely used for the treatment of several types of cancer, especially the solid base.[1] Although it is a very effective antitumor agent, it has many important toxic side effects, including nephrotoxicity, ototoxicity, and neurotoxicity. The most common side effect is nephrotoxicity. Most of the cancer patients treated with Cis have developed serious renal dysfunction.[2] The main reason for serious damage to the kidneys is that Cis accumulates mostly into the proximal tubules of the kidney. Accumulation of Cis into the proximal tubules causes oxidative stress, inflammation, and necrotic and apoptotic cell death, and all these findings lead to the development of acute kidney injury (AKI).[3–5]

It was reported that Cis causes apoptosis by activating the mitochondrial pathways.[6] Cis-treated renal epithelial cells cause BAX protein translocation into mitochondria and activate caspases.[7] Intracellular calcium concentration has been demonstrated to increase after platinum-based anticancer therapy, such as Cis.[8] Calcium levels in the kidney tissues of Cis-treated patients increased by 178% using inductively coupled plasma mass spectrometry analysis.[9]

The superfamily of transient receptor potential (TRP) channels has six subfamilies, of which TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), and TRPP (polycystin) are expressed in mammals. TRPC5 calcium channels were highly expressed in kidney tissues.[10,11] Inhibition or genetic deletion of TRPC5 in podocytes was found to prevent lipopolysaccharides and protamine sulfate-induced nephrotoxicity.[12] The contribution of the TRP channels has not been tested enough in nephrotoxicity models until now due to the lack of potent and specific agonists or antagonists. These channels act as signal converters by altering the membrane potential or intracellular calcium concentration. $Ca^{2+}$ homeostasis is regulated by $Ca^{2+}$ channels including TRPs, and intracellular $Ca^{2+}$ stores.[13] For the specific inhibition of TRPC5 channels, flufenamic acid, SAR7334, SKF96365, $La^{3+}$, and ML204 was tested, but electrophysiological whole-cell recordings proved that clemizole hydrochloride (Clem) is the most potent inhibitor.[14] Despite that, voltage-gated calcium channel blockers such as verapamil and nifedipine were found to ameliorate cyclosporine A-induced renal damage,[15] but the role of TRP channels has not been tested yet for the present indication.

In addition to being a TRP calcium channel blocker, Clem is also known as a histamine H1-receptor antagonist. It has been used as an antihistaminic drug for the treatment of several allergic reactions in the past decades[16–18] until a safer and more specific new generation of antihistaminic compounds was discovered. Currently, clemizole is not used as an antihistaminic agent, but is used only as a feature for blocking TRPC5 cation channels.[19]

AKI is a serious and frequent complication in cancer patients undergoing platinum-based chemotherapy, and the development of strategies for preventing it has primarily focused on decreasing Cis accumulation in renal tubules, oxidative stress, inflammation, and necrotic or apoptotic cell death. Thus, effective therapy for the prevention or protection of nephrotoxicity induced by Cis is still needed. Based on the fact that inhibition of TRPC5 prevents damage to the glomerular filtration barrier, we explored subsequent doses of Clem in an animal model of Cis-induced nephrotoxicity as a therapeutic agent. It is aimed to reveal the possible curative properties of Clem through histological and biochemical analyzes.

## 2 | MATERIALS AND METHODS

### 2.1 | Study design

The study procedure was approved by the Animal Care and Usage Committee of Bezmialem Vakif University and applied according to the institutional guidelines (decision number: 18/229). Thirty-five male Sprague–Dawley rats (Experimental Animal Research Laboratory of Bezmialem Vakif University) were divided into five groups ($n = 7$, for each) as follows: control; Cis; Cis + 1 mg/kg Clem (Cis + Clem 1); Cis + 5 mg/kg Clem (Cis + Clem 5); Cis + 10 mg/kg Clem (Cis + Clem 10).

Cis (catalog no.: 232120) and Clem (catalog no.: SML1447) were purchased from Sigma-Aldrich. Cis dose was adjusted in accordance with the previous studies.[20,21] Cis was administered as a single dose of 8 mg/kg by intraperitoneal injection. Clem was freshly prepared by dissolving it in 10 times diluted stock solution of serum physiologic (SF) + dimethyl sulfoxide (1% DMSO) (1:1) and was administered at 1 mL to the animals. The first dose of Clem was administered to the groups 1 h before Cis administration. Clem was applied for a total of 7 days by oral gavage. Animals were euthanized by cervical dislocation on the 7th day.

### 2.2 | Histological analysis

The left kidney sample was cut into two equal pieces on a vertical plane. The first half was prepared for histological examinations, and both were stored at −80°C. Fixation of tissues was performed in a 10% formalin solution and embedded in paraffin blocks. Five-micrometer sections were stained with Mayer hematoxylin (Merck; catalog no. 05-06004/L) and Shandon eosin Y alcoholic (Thermo Fisher Scientific; catalog no. 6766007), Masson trichrome (Bio-optica; catalog no. 04-010802), and periodic acid Schiff stains (Beslab; BS-0040). Total kidney injury was detected by monitoring the percentage of tubular degeneration (tubular dilation, atrophy, and necrosis), hyaline casts, congestion, hemorrhage, and glomerular collapse in the renal cortex. All parameters were scored as follows: 0 = absent (<10%), 1 = minimal (<10%–25%), 2 = mild (26%–50%), and 3 = severe (>50%), with a maximum score of 15 for each sample.

The cells undergoing apoptosis were additionally examined via the samples stained with a specific in situ apoptosis kit of ApopTag® Peroxidase in Situ Apoptosis Detection Kit (Millipore; catalog no. S7100). Thymus was used as a positive control. Negative control staining was performed according to the manufacturer's instructions.

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Thirteen successive areas in each section were examined for the presence of positive apoptotic cells at ×20 magnification by using the light microscope. Kidney injury was scored in the double-blind study (M. Kumas and M. Esrefoglu) using a Nikon Eclipse i5 light microscope with a Nikon DS-Fi1c camera, and Nikon NIS Elements version 4.0 image analysis systems (Nikon Instruments Inc.).

### 2.3 | Urine urea nitrogen and creatinine levels analysis

Urine urea nitrogen (UUN) and creatinine analysis were conducted on urine samples that were collected for 24 h on the1st and 7th day of the experiment from rats that were housed in metabolic cages. After 3000*g* centrifugation for 15 min, supernatants of all urine samples were collected and stored at −80°C, until the experiment was started. SL1053Ra (Sunlong) and SL0204Ra (Sunlong) commercial kits were used for measuring UUN and creatinine concentrations, respectively, according to the manufacturer's instructions, using the enzyme-linked immunoassay (ELISA) method.

### 2.4 | Oxidative stress markers and neutrophil gelatinase-associated lipocalin analysis

Blood and homogenized tissue samples were transferred into acid citrate dextrose-containing tubes and centrifuged at 3000*g* for 15 min to obtain serum. All samples were stored at −80°C until the experiments were started. Catalase (CAT), malondialdehyde (MDA) in the serum, and neutrophil gelatinase-associated lipocalin (NGAL) levels in urine samples were measured using commercially available kits using the ELISA method. SL1084Ra (Sunlong) was used for CAT, SL0475Ra (Sunlong) for MDA, and SL0526Ra (Sunlong) for NGAL. Total antioxidant status (TAS) and total oxidant status (TOS) were measured in renal tissues using a colorimetric assay according to the manufacturer's instructions (Relassay). The oxidative stress index (OSI) was calculated as follows:

OSI (arbitrary unit) = [(TOS, µmol $H_2O_2$ equivalent/mg protein)/(TAS, µmol Trolox equivalent/mg protein] × 100).

### 2.5 | Western blot analysis

Nephrin, synaptopodin, and Rac family small GTPase 1 (RAC1) expressions were detected with Western blot analysis. Each tissue sample was homogenized in RIPA buffer and proteinase inhibitor cocktail (Merck KGaA). The lysates were centrifuged at 15,000*g* for 10 min at 4°C. The amount of total protein was measured according to the Bradford Coomassie brilliant blue dye method. The protein samples were separated by 12% sodium dodecyl sulfate-polyacrylamide gel and then transferred to polyvinylidene difluoride membranes (Santa Cruz Biotechnology). To avoid nonspecific bindings, all membranes were blocked with 5% powdered skim milk for 1 h. They were further incubated overnight with an IgG-purified nephrin (Bs-10233r; Bioss), synaptopodin (ab117702; Abcam), and RAC1 (Bs-5698R; Bioss) in a solution containing 5% powdered skim milk and 0.05% Triton X-100/TBS. They were washed three times in TBS-T for 10 min each and were further incubated with a peroxidase-conjugated goat anti-rabbit IgG at a concentration of 1 µg/mL in 5% powdered skim milk in 0.05% TBS-T. All samples were also blotted for β-actin antibodies to normalize the protein content by comparing it with other antibody blots. The expression levels of proteins were quantified by using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare) and the bands were captured with an imaging system (Vilber Lourmat Sté). All Western blot experiments were performed in triplicate.

### 2.6 | Statistical analysis

The normality of all data was analyzed with Shapiro–Wilk normality test, due to the sample size which is less than 50. Data that exerted normal distribution (Shapiro–Wilk test, $p \geq 0.05$) were further analyzed with a parametric test of analysis of variance (ANOVA) (Tukey's post hoc honestly significant difference test) for multiple comparisons. *p* Values ≤0.05 were considered as significant. Statistical analyses were conducted using SPSS 20.0 (IBM) and MS Office Excel. GraphPad Prism 6 was used for drawing bar charts.

## 3 | RESULTS

### 3.1 | Histopathological results

The sections from the control group were found normal in histological appearance. The Cis group showed tubular degeneration, congestion, hemorrhage, an abundance of hyaline casts, glomerular degeneration, and glomerular collapse in the renal cortex. The mean histopathological scores (MHSs) and related parameters of the Cis group were higher than those of the control values ($p < 0.001$, for all; Table 1). Tubular degeneration (Figure 1A,B), congestion (Figure 1B), hemorrhage (Figure 1C), hyaline casts (Figure 1B,C), and glomerular collapse (Figure 1A,C) were highly prominent in the Cis group. Tubular degeneration was markedly reduced in all the Clem-treated animals ($p < 0.05$, for all, Figure 2A–C). Administration of 1 and 5 mg/kg Clem successfully decreased hyaline casts, compared to that of the Cis ($p = 0.025$ and $p = 0.008$, respectively). A measure of 10 mg/kg Clem was not as effective as the other doses, and it did not improve the hyaline casts score ($p > 0.05$). Hemorrhage, congestion, and glomerular collapse were observed in the kidney sections of the Cis group when compared to the control group ($p < 0.001$ for both hemorrhage and congestion; $p = 0.039$ for glomerular collapse). But none of the Clem-administered groups was able to improve those histopathological changes in comparison to Cis ($p > 0.05$, for all). While the total MHS of Cis + Clem 1 and Cis + Clem 5 groups were markedly lower than that of the Cis group ($p < 0.001$ for both),

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**TABLE 1** Summary statistics and one-way ANOVA test (Tukey's post hoc HSD) results about the histopathological scores in all groups.

| | Mean ± SD<br>*p* | | | | |
|---|---|---|---|---|---|
| | Control (*n* = 7) | Cis (*n* = 7) | Cis+ Clem 1 (*n* = 7) | Cis + Clem 5 (*n* = 7) | Cis + Clem 10 (*n* = 7) |
| Tubular degeneration (dilation, atrophy, necrosis) | 0.00<br><0.001 | 3.00 ± 0<br>1 | 2.25 ± 0.75<br>0.035 | 1.85 ± 0.38<br><0.001 | 1.71 ± 0.48<br><0.001 |
| Hyaline casts | 0.00<br><0.001 | 2.42 ± 0.78<br>1 | 1.42 ± 0.53<br>0.025 | 1.29 ± 0.49<br>0.008 | 1.71 ± 076<br>>0.05 |
| Congestion | 0.28 ± 0.18<br><0.001 | 2.14 ± 0.69<br>1 | 2.00 ± 0<br>>0.05 | 1.86 ± 0.38<br>>0.05 | 1.71 ± 0.49<br>>0.05 |
| Hemorrhage | 0.00<br><0.001 | 1.57 ± 0.79<br>1 | 1.14 ± 0.38<br>>0.05 | 1.00 ± 0<br>>0.05 | 1.14 ± 0.38<br>>0.05 |
| Glomerular collapse | 0.57 ± 0.53<br>0.039 | 1.71 ± 0.75<br>1 | 1.29 ± 0.95<br>>0.05 | 1.57 ± 0.78<br>>0.05 | 2.14 ± 0.14<br>>0.05 |
| Total Histopathologic Score | 1.00 ± 0.43<br><0.001 | 10.57 ± 1.90<br>- | 8.14 ± 0.89<br>0.009 | 7.71 ± 0.76<br>0.002 | 8.71 ± 1.25<br>>0.05 |

*Note*: All results belong to the cisplatin group compared to the other groups, and *p* values were represented in the columns (SD, *n* = sample size). The total histopathologic score is the sum of the tubular degeneration, hyaline casts, congestion, hemorrhage, and glomerular collapse scores.
Abbreviations: ANOVA, analysis of variance; Cis, cisplatin; Clem, clemizole hydrochloride; HSD, honestly significant difference; SD, standard deviation.

10 mg/kg Clem treatment was not as effective as the others ($p > 0.05$).

An increase in the number of tubular epithelial cells undergoing apoptosis was detected in the Cis group. The mean number of apoptotic cells (MNACs) in the Cis group was markedly higher than that in the controls ($p < 0.001$; Figure 3A–E). MNACs of all the Clem-treated groups were lower than that in the Cis ($p < 0.001$, for all; Figure 3F–H). Results of the statistical analysis of MNAC scores of all groups were presented in Table 2.

### 3.2 | Biochemical results

Cis was able to change all biochemical parameters dramatically when compared to controls (Table 3). While UUN and creatinine levels did not change with Cis administration on the 1st day, they were found to be induced by Cis more than 10-fold on the 7th day (Figure 4). A measure of 1 mg/kg Clem dramatically decreased UUN and creatinine levels compared to those in the Cis group ($p < 0.001$, for all). The levels detected in the Cis + Clem 1 group were very similar to those in the control groups ($p > 0.05$).

Urine NGAL, which directly reflects the kidney injury, was also measured. Cis markedly induced NGAL levels in comparison to the control group ($p < 0.001$; Figure 5). NGAL levels in all the Clem-administered groups were found to be lower than that in the Cis group ($p < 0.05$, for all).

Although it was seen that the levels of UUN, creatinine, and NGAL decreased in the Cis + Clem 5 and Cis + Clem 10 groups when compared to the Cis group, those levels were much higher than those of controls, which were found to be statistically significant ($p < 0.05$, for all).

CAT enzyme levels and TAS dramatically decreased, while TOS and OSI markedly increased in the Cis group ($p < 0.001$). At a dose of 1 mg/kg, Clem acted as a powerful antioxidant, and markedly normalized CAT enzyme level and OSI ($p < 0.001$; Figure 6). However, administration of 5 and 10 mg/kg Clem was not able to normalize oxidative stress parameters compared to controls ($p < 0.001$, for both).

A lipid peroxidation marker of MDA was induced more than 10-fold in the Cis group compared with the control group. MDA levels were found to be almost similar to the control group in the Cis + Clem 1 and Cis + Clem 10 groups ($p < 0.05$, for both; Figure 7).

Western blot analysis was also conducted on kidney tissues for nephrin (Figure 8), synaptopodin (Figure 9), and RAC1 (Figure 10) protein levels. All doses of Clem were able to normalize nephrin, synaptopodin, and RAC1 levels with respect to the Cis group. Levels of nephrin synaptopodin markedly decreased in the Cis group. All doses of Clem significantly induced the expression of both ($p < 0.05$, for all). Expression of RAC1, an indicator for podocyte injury, was induced by Cis. It markedly decreased in all the Clem-treated groups ($p < 0.001$, for all).

## 4 | DISCUSSION

In the present study, dose-dependent protective effects of Clem against Cis-induced nephrotoxicity were examined in rats. Clemizole was a histamine H1-receptor antagonist drug, first developed in the 1950s.[22,23] It was presented as a strong antihistaminic effect with low toxicity in animal experiments.[16] It has been used as an antihistaminic drug for the treatment of several allergic reactions and pruritus of dermatoses in the past

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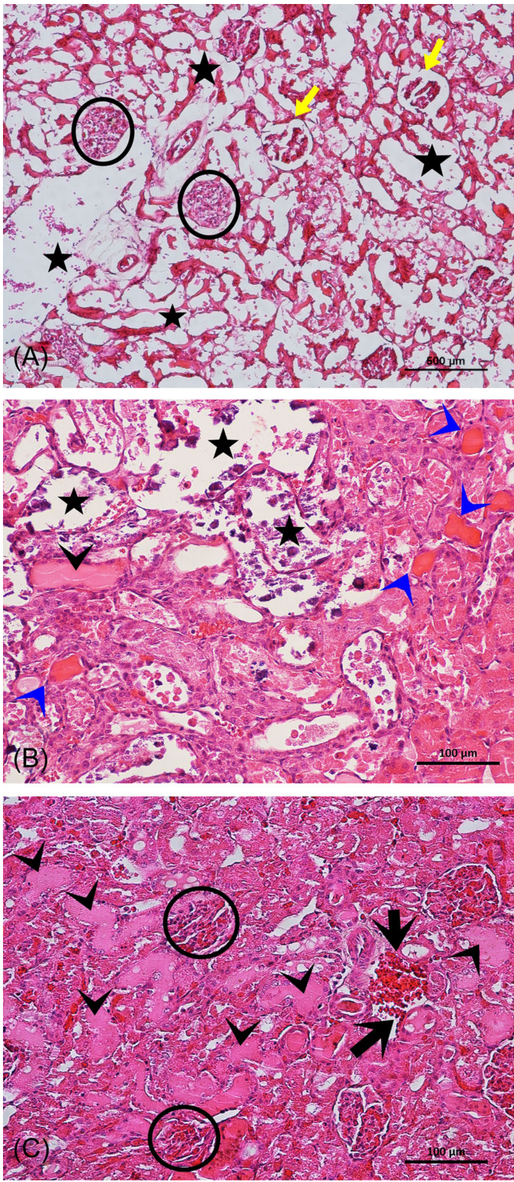


FIGURE 1 Light microscopic views of renal cortex in the cisplatin (Cis) group with tubular degeneration indicated by tubular dilatation, atrophy, and necrosis (A, B; asterisks), congestion (B; blue arrowheads), hyaline casts (B, C; dark arrowheads), glomerular degeneration (A; yellow arrow) and collapse (A, C; in circles), and hemorrhage (C; dark arrows) are observed in the Cis group. Note the cellular debris within the lumen of the tubules (hematoxylin and eosin; ×20).

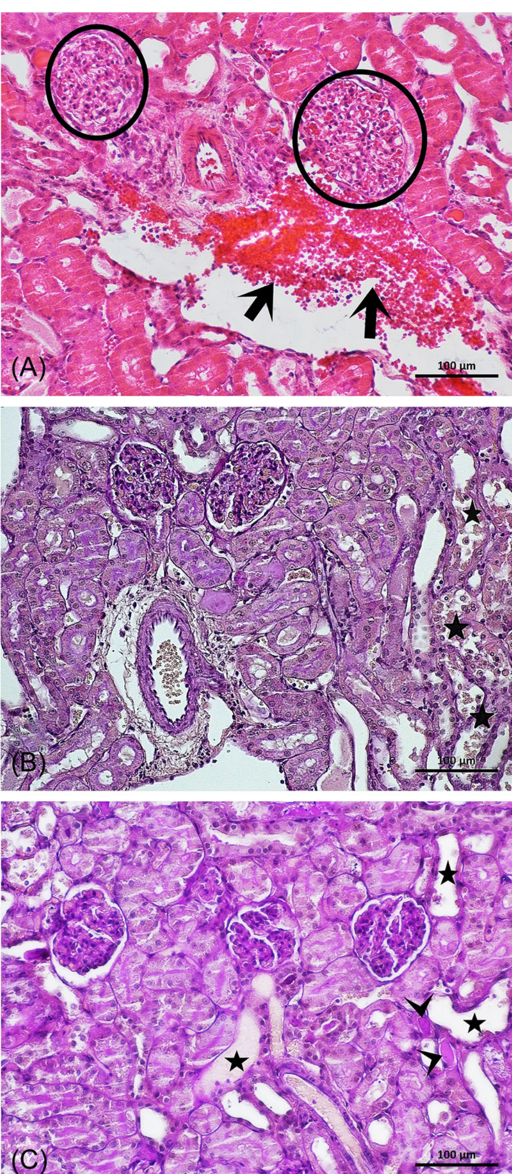


FIGURE 2 Light microscopic view of the renal cortex obtained from Cis + Clem 1 (A), Cis + Clem 5 (B), and Cis + Clem 10 (C) groups. Dose-dependent improvement in all the histological alterations induced by cisplatin (Cis) is observed in clemizole hydrochloride (Clem)-administered groups. Prominent glomerular collapse (in circles) and hemorrhage (dark arrows) are observed in the Cis + Clem 1 group (A; hematoxylin and eosin stain; ×20). Glomerulus and tubules in general seem normal; tubules undergoing degeneration are marked with asterisks in the Cis + Clem 5 group (B, periodic acid Schiff stain; ×40). Tubular degeneration (asterisks) and mild hyaline casts (dark arrowheads) are seen in the Cis + Clem 10 group (C, periodic acid Schiff stain; ×40).

decades.[17,18,24] The clemizole–penicillin combination was widely employed for treating syphilis.[25–27] Nowadays, clemizole becomes popular again with not only its antihistaminic features but also the blocking effects of TRPC5 cation channels, specifically. On the other hand, Cis is widely used in the treatment of several malignancies as an antitumoral drug.[20] Cis has serious side effects, the most common one of which is nephrotoxicity.[28] Due to its high renal reabsorption, it can impair kidney function very rapidly.[29] It shows serious toxic effects on renal tissues and causes AKI. Accumulation of cisplatin in the tubular epithelium, especially the S3 segment of proximal tubules, markedly causes loss of brush borders and epithelial cell necrosis,[30] dramatically decreases glomerular filtration,[20] increases oxidative stress,[31] apoptosis,[32] and lipid peroxidation.[33]

Glomerular damage induced by Cis is seen in kidney tissues of patients and experimental animals. The literature provides data about

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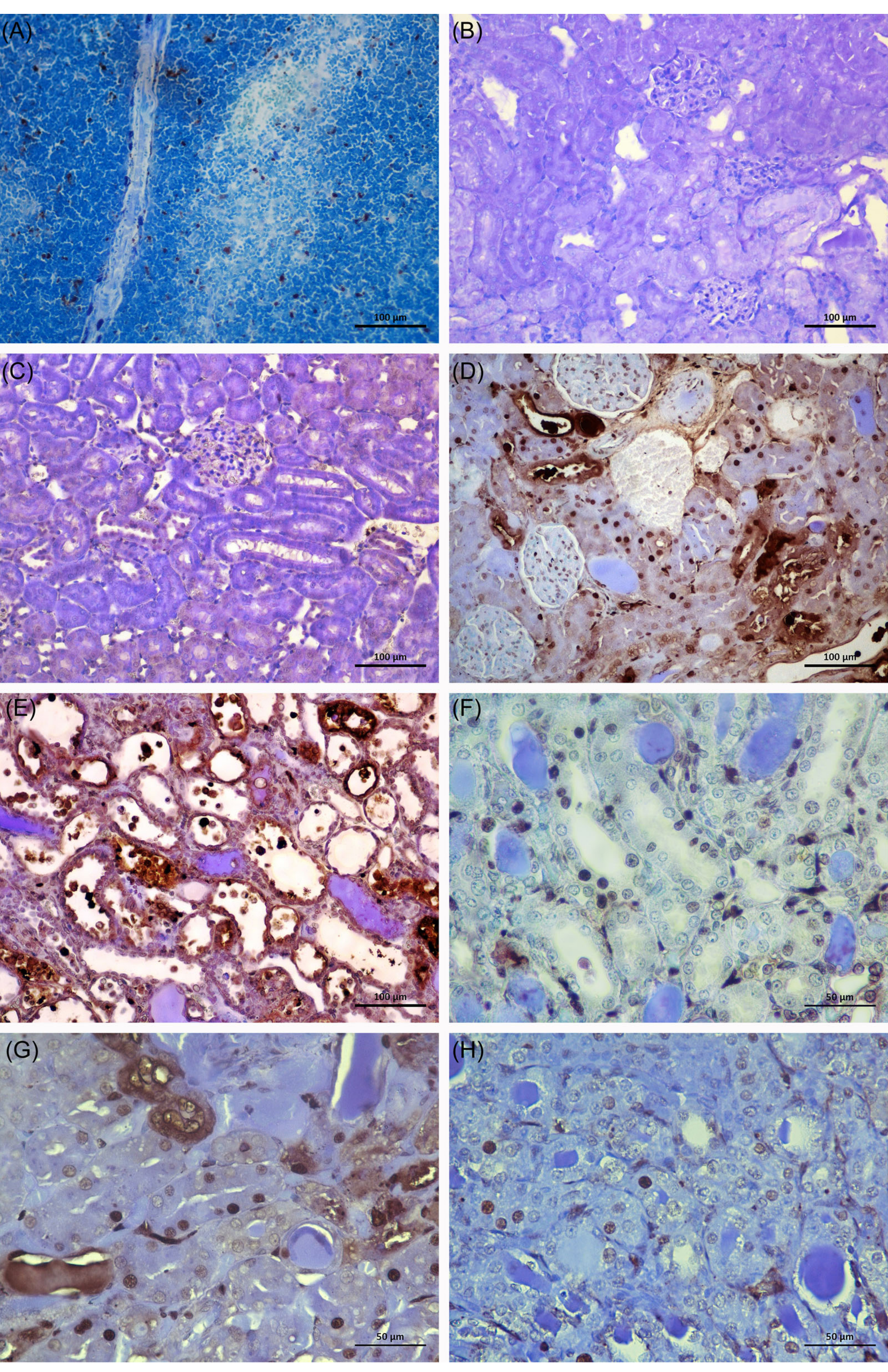


**FIGURE 3** Sections stained with ApopTag® Peroxidase in Situ Apoptosis Detection Kit obtained from all groups. Cisplatin caused apoptotic cell death, and all doses of clemizole hydrochloride showed antiapoptotic effects (A: positive control; thymus, B: Negative control, C: Control group; D, E: Cis group; F: Cis + Clem 1 group; G: Cis + Clem 5 group; H: Cis + Clem 10 group). Cis, cisplatin; Clem; clemizole hydrochloride.

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**TABLE 2** Summary statistics and one-way ANOVA test (Tukey's post hoc HSD) results about the number of apoptotic cells in all groups.

| **Mean ± SD**<br>***p*** | | | | |
|---|---|---|---|---|
| **Control (*n* = 7)** | **Cis (*n* = 7)** | **Cis + Clem 1 (*n* = 7)** | **Cis + Clem 5 (*n* = 7)** | **Cis + Clem 10 (*n* = 7)** |
| 5.31 ± 2.32<br><0.001 | 141.61 ± 40.56<br>- | 75.00 ± 25.45<br><0.001 | 99.83 ± 29.5<br><0.001 | 91.02 ± 26.11<br><0.001 |

*Note*: All results belong to the cisplatin group compared to the other groups, and *p* values were represented in the columns (SD, *n* = sample size).

Abbreviations: ANOVA, analysis of variance; Cis, cisplatin; Clem, clemizole hydrochloride; HSD, honestly significant difference; SD, standard deviation.

**TABLE 3** Summary statistics and one-way ANOVA test (Tukey's post hoc HSD) results about biochemical analysis in all groups.

| | **Mean ± SD**<br>***p*** | | | | |
|---|---|---|---|---|---|
| | **Control (*n* = 7)** | **Cis (*n* = 7)** | **Cis + Clem 1 (*n* = 7)** | **Cis + Clem 5 (*n* = 7)** | **Cis + Clem 10 (*n* = 7)** |
| UUN on the 1st day (mg/dL) | 124.22 ± 16.09<br>>0.05 | 148.96 ± 19.55 | 142.33 ± 17.56<br>>0.05 | 121.25 ± 15.820.042 | 111.23 ± 14.51<br>0.003 |
| UUN on the 7th day (mg/dL) | 126.76 ± 12.39<br><0.001 | 1603.07 ± 66.95 | 145.40 ± 12.44<br><0.001 | 1300.42 ± 47.52<br><0.001 | 1193.04 ± 43.59<br><0.001 |
| Creatinine on the 1th day (mg/dL) | 7.05 ± 0.14<br><0.001 | 6.19 ± 0.17 | 6.09 ± 0.17<br>>0.05 | 5.83 ± 0.41<br>>0.05 | 5.54 ± 0.16<br><0.001 |
| Creatinine on the 7th day (mg/dL) | 6.92 ± 0.10<br><0.001 | 81.89 ± 2.17 | 6.30 ± 0.16<br><0.001 | 54.77 ± 2.40<br><0.001 | 53.81 ± 2.73<br><0.001 |
| NGAL (ng/mL) | 2.18 ± 0.41<br><0.001 | 11.80 ± 0.51 | 2.32 ± 0.26<br><0.001 | 9.86 ± 1.16<br>0.002 | 10.07 ± 1.26<br>0.007 |
| CAT (pg/mL) | 45.90 ± 2.62<br><0.001 | 15.28 ± 2.33 | 45.21 ± 2.11<br><0.001 | 25.10 ± 3.23<br><0.001 | 29.54 ± 3.89<br><0.001 |
| MDA (ng/mL) | 28.40 ± 5.30<br><0.001 | 381.82 ± 18.45 | 29.85 ± 4.99<br><0.001 | 13.52 ± 1.91<br><0.001 | 24.74 ± 6.77<br><0.001 |
| TAS (mmol Trolox equivalent/L) | 1.39 ± 0.06<br><0.001 | 0.31 ± 0.04 | 1.27 ± 0.06<br><0.001 | 0.33 ± 0.06<br>>0.05 | 0.33 ± 0.05<br>>0.05 |
| TOS (µmol $H_2O_2$ Trolox equivalent/L) | 8.32 ± 0.40<br><0.001 | 12.11 ± 0.62 | 7.76 ± 0.46<br><0.001 | 9.93 ± 0.61<br><0.001 | 10.91 ± 0.74<br>0.008 |
| RAC1 | 0.97 ± 0.05<br><0.001 | 4.75 ± 0.10 | 0.82 ± 0.04<br><0.001 | 2.61 ± 0.15<br><0.001 | 0.84 ± 0.04<br><0.001 |

*Note*: All results belong to the cisplatin group compared to the other groups, and *p* values were represented in the columns (SD, *n* = sample size).

Abbreviations: ANOVA, analysis of variance; CAT, catalase; Cis, cisplatin; Clem, clemizole hydrochloride; HSD, honestly significant difference; MDA, malondialdehyde; NGAL, neutrophil gelatinase-associated lipocalin; RAC1, Rac family small GTPase 1; SD, standard deviation; TAS, total antioxidant capacity; TOS, total oxidant status; UUN, urine urea nitrogen.

glomerular damages including glomerular collapse, Bowman's space thickened, and so forth, caused by Cis.[33,34] We observed similar histopathological alterations in kidney sections of the Cis group.

ROS has been shown to activate death receptor, mitochondrial, and ER-mediated apoptotic pathways in a calcium-dependent manner.[14] It was reported that a new apoptotic–necrotic hybrid pathway, called necroptosis, has gained attention in Cis-induced AKI.[35] Not only apoptotic but also some necrotic parameters were found to be exacerbated by the administration of Cis. According to our results, Cis obviously induces apoptosis and necrosis consecutively in the kidney. The contribution of TRPC5 calcium channels to that pathologic cascade was well-documented. It can be speculated that all doses of Clem exerted antiapoptotic effect in Cis-induced apoptosis.

TRP channels were reported to be expressed and function in a great variety of multicellular organisms, including worms, fruit flies, zebrafish, mice, and humans.[36] It was also shown that TRPC, TRPV, TRPM, and TRPP subfamilies are expressed in rat and human kidneys.[37] TRP channels regulate cellular processes, including cell volume, vascular tone and permeability, proliferation, angiogenesis,

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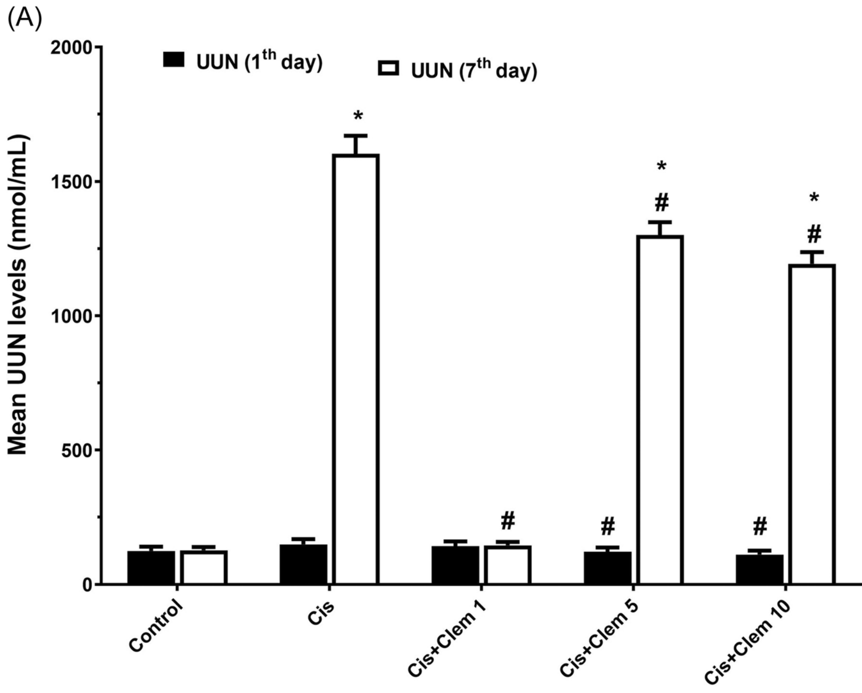


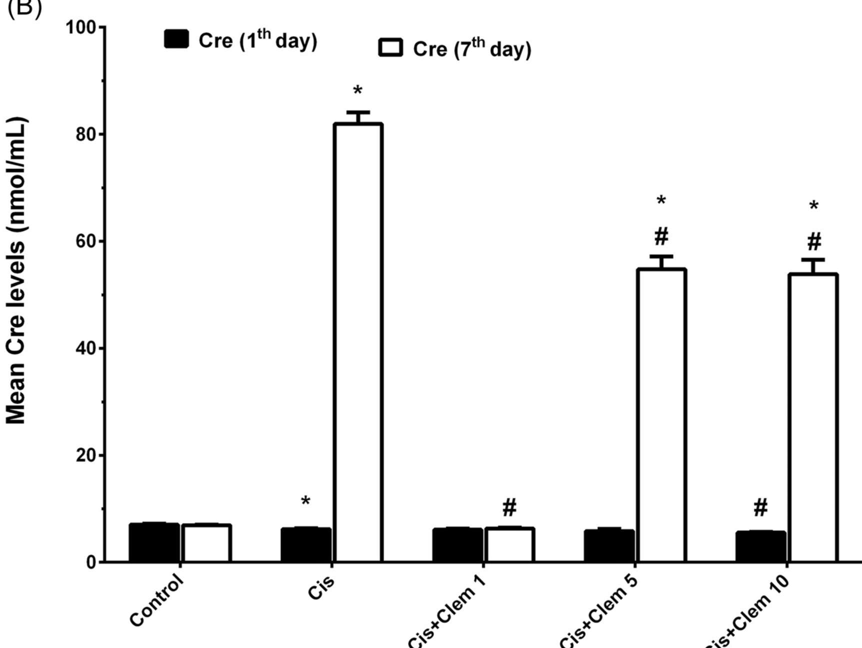


**FIGURE 4** (A) Mean urine urea nitrogen (UUN) and creatinine levels in all groups. (B) Mean creatinine levels in all groups. UUN and creatinine levels severely increased in the cisplatin groups on the 7th day, whereas the levels on the 7th day were markedly decreased in clemizole hydrochloride-treated groups. ($^*p < 0.05$ vs. control group, $^\#p < 0.05$ vs. cisplatin group, one-way analysis of variance [ANOVA], post hoc Tukey's test). Cis, cisplatin; Clem, clemizole hydrochloride; Cre, creatinine.

secretion, and apoptotic cell death.[38] Among subfamilies of TRP, TRPC is found to be widely distributed in the kidney. TRPC1 was expressed in mesangial cells, while TRPC3 in podocytes, distal convoluted tubules and collecting ducts, TRPC5 in podocytes and juxtaglomerular cells, and TRPC6 in podocytes and collecting ducts.[39] Inhibition of TRPC5 in podocytes was reported to prevent glomerular filtration barrier damage in lipopolysaccharide and protamine sulfate-induced kidney injury in the mouse.[12] Concurrent use of calcium channel blockers, such as verapamil and nifedipine was found to prevent the biochemical, morphological, and functional derangements induced by cyclosporine A.[15] We have got a very similar protection profile of the TRPC5 channel blocker, especially in a lower concentration. In the current study, lower doses of Clem significantly ameliorated the morphological and functional derangements caused by Cis via improving histopathological alterations and decreasing apoptotic and necrotic cell death.

Even though the sources of intracellular calcium were several, as mentioned previously, it could be speculated that calcium has a pivotal role in survival such as vesicle secretion, muscle contraction, gene transcription, cytoskeletal structure, and so on, as well as the pathologic processes of oxidative, fibrotic, and inflammatory manifestations of the cells.[40] It was reported that the Cis administration

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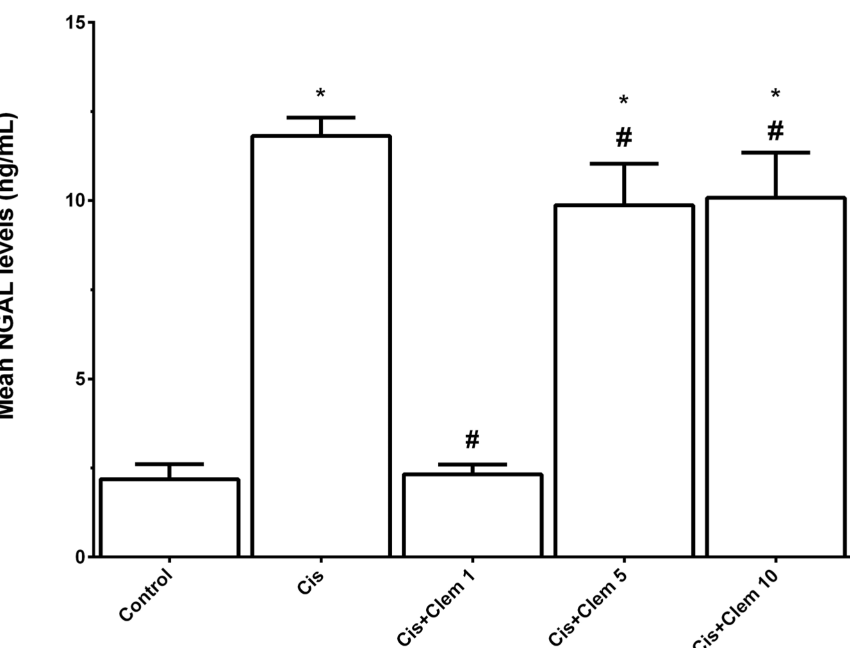


**FIGURE 5** Mean urine neutrophil gelatinase-associated lipocalin (NGAL) levels in all groups. Mean NGAL level in the cisplatin group was markedly higher than those in the clemizole hydrochloride -treated groups (*$p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. cisplatin group, one-way analysis of variance [ANOVA], post hoc Tukey's test). Cis, cisplatin; Clem, clemizole hydrochloride.

is associated with impaired mitochondrial function, induced oxidative stress, and decreased antioxidant enzymes.[41] Upon receptor-mediated endocytosis, Cis is hydrolyzed into a positively charged molecule, as an electrophile, it directly disrupts the action of mitochondrial complexes, leading to increased production of ROS and decreased ATP production.[3] Highly compatible results were obtained in our study, with the MDA, CAT, TAS, TOS, and OSI. MDA is an end product of the lipid peroxidation process of mainly membrane lipids; thus, it is used as one of the markers of membrane damage. Since reactive oxygen species degrade polyunsaturated lipids forming MDA, it is also one of the clues of oxidative stress. Total oxidant and antioxidant levels of the tissue is also a marker for evaluating oxidative stress. In our study according to MDA levels, membrane damage of the Cis + Clem 5 group is higher than the Cis + Clem 1 and Cis + Clem 10 groups. MDA levels of Cis + Clem 1 and Cis + Clem 10 are similar to each other. MDA was detected in the serum but not in the tissue, so the results show us the membrane damage status of all the organs but not of the kidneys alone. When we look at the histopathological scoring, we see that the levels of the treatment groups are similar to each other. Especially when we look at the histopathological scoring results in terms of tubular degeneration, hyaline cats, and glomerular collapse, which are related to membrane damage, we see that the mean level is 6.85 in the Cis group, 4.85 in the Cis + Clem 5 group, and 5.84 in the Cis + Clem 10 group. So the Cis + Clem 10 group is not better than the Cis + Clem 5 group. According to the OSI, again, the most effective dose is 1 mg/kg.

It has been reported in experimental and clinical studies that Cis causes an elevation of both UUN and creatinine levels.[21,42–44] Increased UUN and creatinine levels indicate renal failure.[21,42–44] NGAL, a glycoprotein, which was first isolated from azurophilic granules of human neutrophils, accumulates in the renal tubules and is secreted into the urine after AKI.[34] Both serum and urine NGAL protein levels have been recently accepted as a biomarker of AKI.[42–44] In the present study, NGAL, UUN, and creatinine levels are consistent with the histopathological results. Cis seriously induced NGAL, UUN, and creatinine levels, while those levels were markedly normalized after Clem administration at a dose of 1 mg/kg.

We also aimed to evaluate the effects of Cis on podocyte functioning, which is the most important process in glomerular filtration. For that purpose, we detected the levels of nephrin, synaptopodin, and RAC1 by the Western blot analysis method. Nephrin, a podocyte protein and a major component of the slit diagram junctional complex, is necessary for the proper functioning of the renal filtration barrier.[45] Downregulation of nephrin has been observed in many human glomerular diseases such as minimal change disease,[46] membranous nephropathy, membranoproliferative glomerulonephritis, IgA nephropathy, lupus nephritis,[47] diabetic kidney disease,[48,49] and pre-eclampsia.[50] Nephrin deficiency may be considered a pathologic feature of glomerular injury, especially as a marker for acute podocyte injury. It was stated that cisplatin leads to a decrease in the level of nephrin in renal tissues.[45] Synaptopodin is expressed in the foot processes of podocytes.[51] A decrease in the level of synaptopodin in various kidney diseases is associated with structural and functional changes in podocytes and changes in the production of proteinuria. It was shown that synaptopodin can limit the expression of TRPC6.[52] TRPC6 is a podocyte pore membrane protein on the podocyte surface to reduce proteinuria. Actin filaments which are more abundant in the secondary processes of the podocytes make an important contribution to the filtration barrier.[52,53] It was shown that TRPC5 promoted the degradation of synaptopodin, leading to cytoskeletal remodeling both in vitro and in vivo, in animal models of nephrotoxic diseases.[12]

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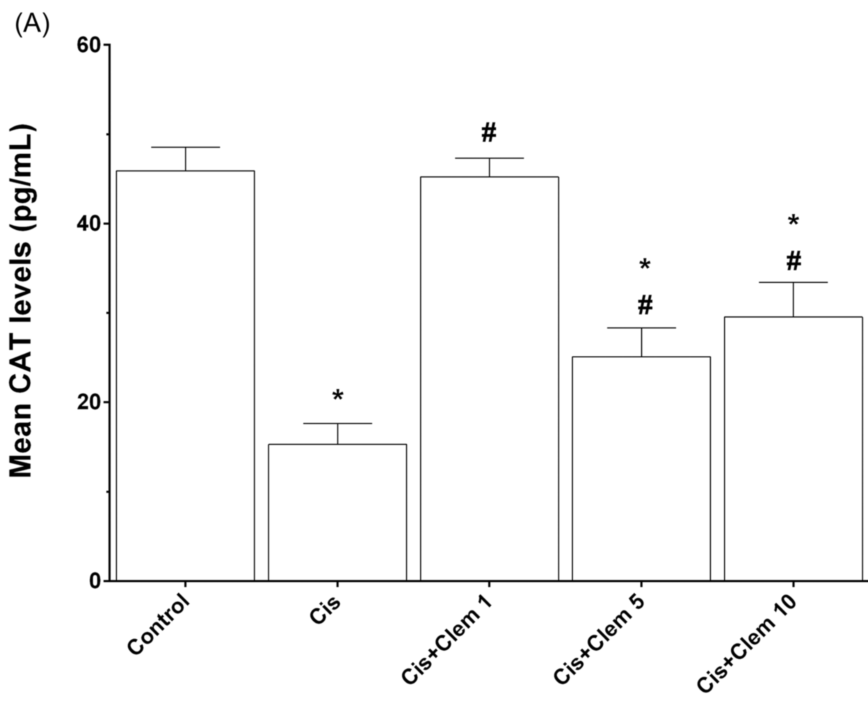


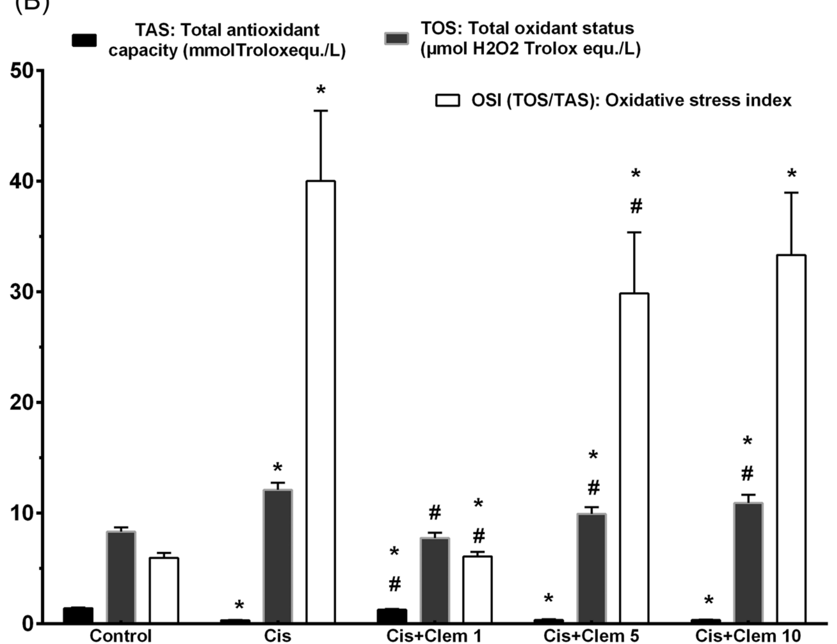


**FIGURE 6** (A) Mean catalase (CAT) levels in all groups. (B) Mean total antioxidant status (TAS), total oxidant status (TOS) levels, and oxidative stress index in all groups. Cisplatin treatment resulted in oxidative stress via decreasing catalase and TAS levels and increasing TOS level and oxidative stress index. Cisplatin-induced oxidative stress markedly improved after all doses of clemizole hydrochloride treatment ($^{*}p < 0.05$ vs. control group; $^{\#}p < 0.05$ vs. cisplatin group, one-way analysis of variance [ANOVA], post hoc Tukey's test). Cis, cisplatin; Clem, clemizole hydrochloride.

TRPC5 and TRPC6 channels are reported as antagonistic regulators of actin remodeling in podocytes because it was detected TRPC6 in a complex with the GTPase RhoA and found TRPC5 in a complex with RAC1.[54] Increased TRPC5 activity is associated with proteinuric disease progression. Genetic deletion or pharmacologic inhibition of TRPC5 was reported to protect mice from albuminuria. These data revealed that the $Ca^{2+}$-permeable channel TRPC5 is an important determinant of albuminuria and identifies TRPC5 inhibition as a therapeutic strategy for the prevention or treatment of proteinuric kidney disease. Podocyte-specific TRPC5/RAC1 signaling thus emerges as the key, specific, and specifically inducible pathway necessary for acute, and possibly reversible, filter barrier disruption.[12] TRPC5 was also found to drive RAC1 activity, and RAC1 conversely activates TRPC5 to mediate disease-associated podocyte motility. RAC1 was reported to be very crucial for the pedicel functioning, and formation of lamellipodia.[55] It also regulates the cell cycle, cell motility, and cell survival. Overexpression of RAC1 in several malignancies leads to the activation of multiple signaling pathways, including uncontrolled proliferation, invasion, and metastasis.[56] Thus, inhibition of RAC1 suppresses inflammasomes in podocytes, and thus prevents foot process effacement, in case of podocyte injury and glomerular sclerosis.[55–57] RAC1 activity depression was presented as a crucial therapeutic strategy for proliferative disorders, such as malignancies.[56]

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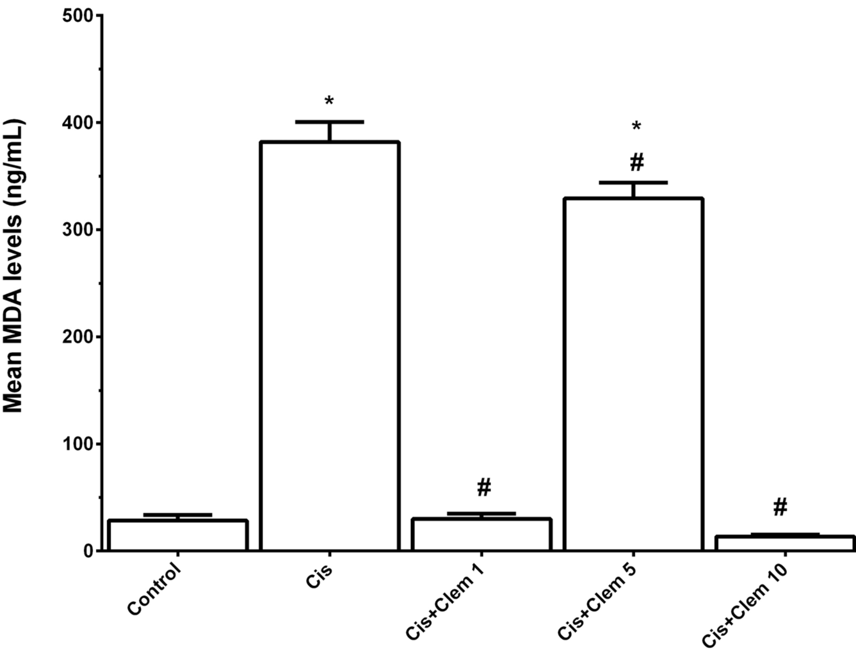


**FIGURE 7** Mean malondialdehyde (MDA) levels in all groups. Cisplatin caused lipid peroxidation via increasing MDA levels; all doses of clemizole hydrochloride normalized MDA levels. ($^*p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. cisplatin group, one-way analysis of variance [ANOVA], post hoc Tukey's test). Cis, cisplatin; Clem, clemizole hydrochloride.

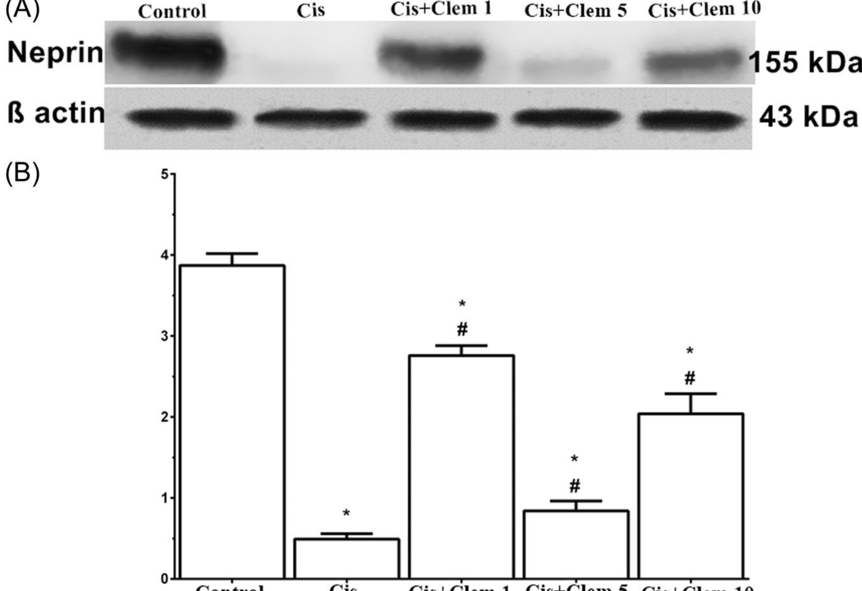


**FIGURE 8** (A) Western blot analysis of nephrin protein levels in all groups. (B) Mean nephrin protein levels in all groups. Nephrin expression was significantly lower in the cisplatin group, whereas those of protein expressions in clemizole hydrochloride-treated groups markedly increased ($^*p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. cisplatin group, one-way analysis of variance [ANOVA], post hoc Tukey's test). Cis, cisplatin; Clem, clemizole hydrochloride.

As mentioned in previous sections, the roles and clinical importance of synaptopodin, RAC1, and nephrin in various diseases of kidney were well documented and, our data was compatible with previous results. Administration of Clem at doses 1 and 10 mg/kg successfully normalized all three indicator protein expressions induced by Cis, and pharmacologic inhibition of $Ca^{2+}$-permeable channel TRPC5 by Clem resulted in downregulation of Rac1 and upregulation of nephrin and synaptopodin. Although at a dose of 10 mg/kg Clem seems to be as effective as the 1 mg/kg dose, when all parameters are considered, the most effective therapeutic dose is 1 mg/kg.

In conclusion, we suggest that Clem, a potent inhibitor of TRPC5 calcium channels, is highly effective to improve histopathological and biochemical changes in Cis toxicity via blocking TRPC5 calcium channels. A dose of 1 mg/kg Clem protected the glomerular filtration barrier by adjusting the nephrin, synaptopodin, and RAC1 expressions; reactivated the antioxidant system; and decreased apoptotic cell death via limiting calcium accumulation into the renal tubules. According to these data, we suggest that Clem at a dose of 10 mg may be used as a potential therapeutic agent against renal ischemic injury, but the most effective dose seems to be 1 mg/kg. Further studies need to be conducted to sort out the contribution of numerous TRP and voltage-gated calcium channels, as well as intracellular calcium stores in nephrotoxic pathophysiology.

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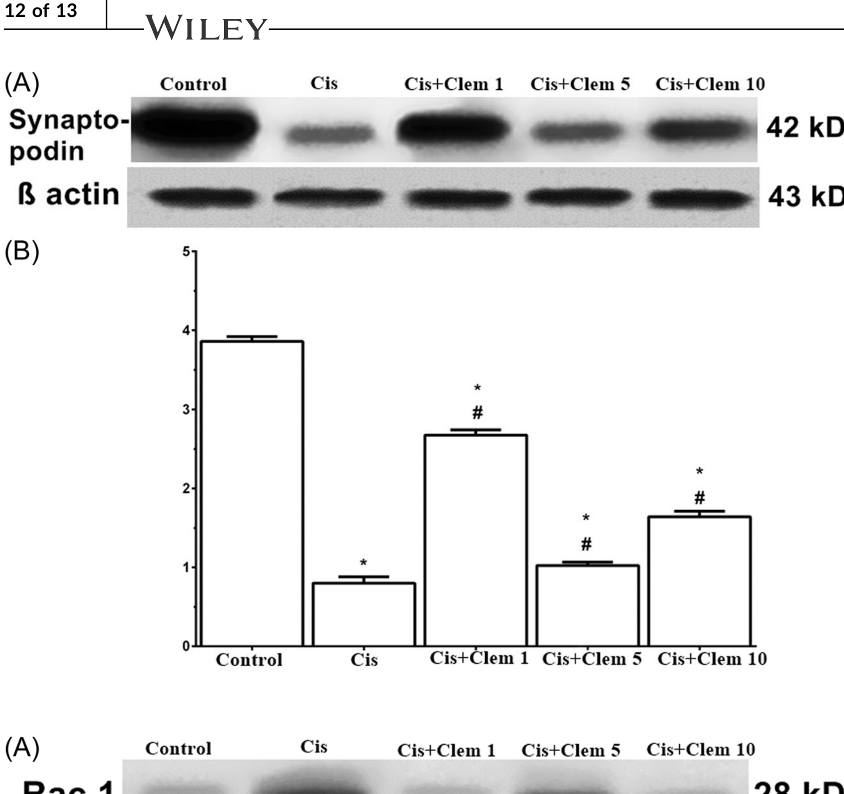


**FIGURE 9** (A) Western blot analysis of synaptopodin protein levels in all groups. (B) Mean synaptopodin protein levels in all groups. Synaptopodin expression dramatically decreased in the cisplatin group, whereas those of protein expressions in the clemizole hydrochloride-treated groups markedly increased (*$p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. cisplatin group, one-way analysis of variance [ANOVA], post hoc Tukey's test). Cis, cisplatin; Clem, clemizole hydrochloride.

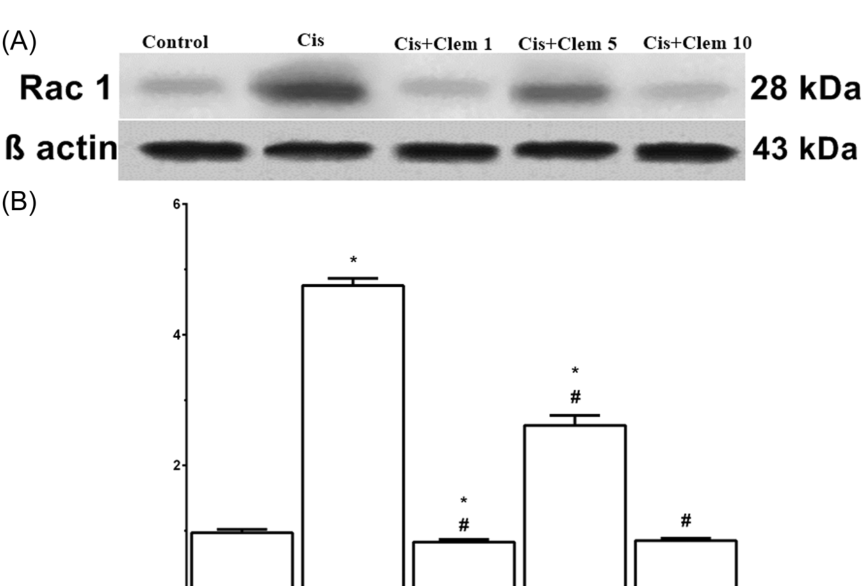


**FIGURE 10** (A) Western blot analysis of Rac family small GTPase 1 (RAC1) protein levels in all groups. (B) Mean RAC1 protein levels in all groups. RAC1 expression in the cisplatin group was dramatically higher than the clemizole hydrochloride-treated groups (*$p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. cisplatin group, one-way analysis of variance [ANOVA], post hoc Tukey's test).


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## DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article. Derived data supporting the findings of this study are available from the corresponding author on request.


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