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Original Article

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Effect of Epigallocatechin Gallate on Cisplatin-Induced Nephrotoxicity in Rats

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ABSTRACT

Objectives: Nephrotoxicity caused by CDDP is attributed to an increase in kidney oxidative stress, which restricts the therapeutic application of CDDP. EGCG is a very strong green tea-derived antioxidant. To investigate the effect of epigallocatechin gallate (EGCG) on cisplatin (CDDP)-induced lipid peroxidation and nephrotoxicity in rats.

Methods: Twenty-eight male Wistar rats (8 weeks, 200-215 g) were used in the research and were separated into 4 equal groups: (1) control rats, (2) EGCG (100 mg/kg, daily) control rats, (3) CDDP-injected rats, CDDP group (7 mg/kg, i.p., single dose), and (4) EGCG-treated (100 mg/kg, daily) plus CDDP-injected rats (EGCG + CDDP group).

Results: There was a substantial rise in malondialdehyde (MDA) levels in the CDDP-injected rats ($P < .05$). The EGCG prevented the rise of the MDA ($P < .05$). In CDDP-injected rats, renal Bax protein expression was substantially higher compared with control rats, and EGCG therapy significantly decreased Bax protein levels ($P < .05$). In EGCG + CDDP-administered rats, Bcl-2 protein expression was higher than in CDDP-injected rats ($P < .05$). In the EGCG + CDDP group, the expression of renal heat shock protein 60 (Hsp60) and heat shock protein 70 (HSP70) was significantly lower compared to CDDP-alone injected rats ($P < .001$). CDDP-induced renal histopathological changes were significantly improved with EGCG.

Conclusion: CDDP produces oxidative stress and renal damage. The EGCG that reduces oxidative stress and changes the expression of Bax and Bcl-2 proteins may potentially have a significant role in the prevention of CDDP-induced renal injury.

Keywords: Cisplatin, nephrotoxicity, epigallocatechin gallate, malondialdehyde, Bax/bcl-2, Hsp60–70

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INTRODUCTION

Cisplatin (*cis*-diamminedichloroplatinum(II) or CDDP) is a potent and valuable chemotherapy agent that is used in the treatment of various malignancies.[1] Nephrotoxicity is one of the most important side effects associated with CDDP treatment.[2] The effect of CDDP is cumulative and dose-dependent. At low doses (≤40 mg/m^2), renal damage is generally reversible. At higher doses (2 or 75 mg/m^2), renal damage can be acute and irreversible. While the effects of nephrotoxicity observed after CDDP treatment are not known clearly, the widely accepted view is that renal injury arises via a mechanism associated with renal peroxidation.[3]

In a healthy organism, free oxygen radicals (reactive oxygen species, ROS) are produced during normal metabolism. An increase in free ROS production and/or failure of the antioxidant system can result in oxidative damage. Cells possess protective antioxidant mechanisms that prevent, limit, or partially repair oxidative damage.[4] Antioxidants present in the diet include vitamin C, vitamin E, and carotenoids. Other plant components, including polyphenols, have also been shown to possess antioxidant potential.[5] Tea is a beverage rich in phenolic substances. Of the polyphenols in tea leaves, 3/4 are flavanols; and 60-70% of these flavanols are comprised of (−)-epigallocatechin-3-gallate (EGCG).[6] EGCG,

considered to be the most abundant in green tea, is an important in vitro eliminator of ROS and, because of its effects on transcription factors and enzymatic activity, it has antioxidant and anti-inflammatory roles.[7–10]

The purpose of this research is to study the effects of EGCG on the expression of certain proteins (Hsp60, Hsp70, Bax, Bcl-2) that play a key role in cellular functions in CDDP-treated rats. Moreover, the effects of EGCG on histopathological changes in the kidney, renal function tests (serum urea, creatinine levels), and oxidative stress (malondialdehyde (MDA) levels) were also investigated.

METHODS

Animals

After receiving permission from the Animal Research Ethics Committee of Fırat University (Ethics committee approval number: 6), this analysis was carried out in compliance with the normative ethical guidelines for experimental animal research using male Wistar albino rats weighing between 200 and 215 g (*n* = 28, 8 weeks old) obtained from the experimental research center of the university. Throughout the study, the animals were allowed access to food and water ad libitum. Food was provided in special steel containers and water as regular tap water in steel-ball bearing spouts. Experimental animals were fed with pellet food prepared specially at a pellet food factory. The environment where the experimental animals were kept was monitored to maintain a temperature of 22 ± 2°C and a 12 hours : 12 hours light : dark cycle.

Study Protocol

The CDDP (Sigma Chemical Co, USA) was administered in a single dose of 7 mg/kg in physiological saline (1 mL/100 g/kg i.p.) on the third day of the study by i.p., injection and kidney injury was induced. EGCG was diluted in physiological saline at 100 mg/kg (0.89%) through gavage,[11] and administered once a day over a period of 12 days, starting 2 days prior to the CDDP application and for 10 days after the application.

Main Points

- Cisplatin (CDDP) treatment leads to an increase in pro-apoptotic Bax protein and Bax-mediated Hsp proteins in kidney tissue and also to an increase in serum malondialdehyde (MDA) level, which is an indicator of oxidative stress.
- The epigallocatechin gallate (EGCG) has antioxidant activity and is found in green tea leaves.
- Pro-apoptotic proteins (Bax and Hsp 60H0) and MDA levels decrease and apoptosis-inhibiting protein (Bcl-2) increases in rats treated with CDDP and EGCG.
- EGCG decreases CDDP nephrotoxicity through its antioxidant activity.

Rats were distributed randomly to 4 groups

1. Control group (*n* = 7): Rats not treated with CDDP, treated with i.p., isotonic saline solution of equal volume to CDDP (1 mL/kg/day) on the third day and fed a basal diet.
2. EGCG group (*n* = 7): Rats not treated with CDDP, treated with i.p., isotonic saline solution of equal volume to CDDP (1 mL/kg/day) on the third day, and administered EGCG (TEAVIGO, DSM Co, Switzerland) (100 mg/kg) starting 2 days prior to the CDDP application and for 10 days after the application.
3. CDDP group (*n* = 7): Rats treated with CDDP (CDDP; Sigma Chemical Co, USA) (7 mg/kg) in 0.9% saline (1 mL/100 g/kg i.p.).
4. EGCG + CDDP group (*n* = 7): Rats treated with CDDP, administered EGCG (100 mg/kg) starting 2 days prior to the CDDP application and for 10 days after the application.

Data Collection

Ten days after the CDDP application, rats were decapitated under anesthesia to obtain tissue specimens for histopathological and Western blot analyses. The specimens were stored at −80°C until Western blot analyses were performed. The kidneys were perfused with phosphate-buffered solution (PBS; 0.15 M NaCl and 0.01 M sodium phosphate buffer, pH 7.4) through the aorta and removed for histological assessment. Blood was obtained to test serum levels of urea nitrogen and creatinine. Samples of blood were centrifuged for 10 min at 300 × g to isolate the serum. A biochemical analyzer was used to calculate the levels of serum urea nitrogen, creatinine, and MDA (Olympus AU-660, Japan).

For the measurement of protein expression using Western blot analysis, kidney tissue was homogenized (1 : 10, w/v) in buffer solution [10 mM Tris-HCl, pH 7.4, 0.1 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μM soy as trypsin inhibitor (soluble powder; Sigma, St. Luis, MO, USA)]. At 15 000 × g, 4°C for 30 minutes, tissue homogenates were centrifuged. Supernatants were transferred to new tubes. In conjunction with the Lowry protocol, protein concentrations were measured using a protein measuring kit (Sigma, St. Luis, MO, USA). The supernatants were added to the sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis buffer containing 2% β-mercaptoethanol. For electrophoresis, equivalent quantities of protein (20 μg) were isolated by SDS-PAGE gel. These were then moved to nitrocellulose membranes (Schleicher and Schuell Inc, Keene, NH, USA).[12] Nitrocellulose blots were washed twice in PBS for 5 min and allowed to settle in 1% bovine serum albumin for 1 h before the primary antibody was added. The main antibody (anti-Bax, anti-Bcl-2, anti-Hsp60, anti-Hsp70; Santa Cruz Biotechnology Inc, CA, USA) was dissolved in the same 0.05% Tween-20 buffer solution in 1 : 1000 ratio. Nitrocellulose membranes were incubated overnight with protein antibodies at 4°C. Blots were rinsed and incubated with *horseradish peroxidase-conjugated goat anti-rabbit* or *anti-mouse IgG* (Santa Cruz Biotechnology Inc, CA, USA). Using substrates of diaminobenzidine and H_2O_2, unique bindings were

Table 1. Serum Urea Nitrogen, Creatinine, and MDA Levels of the Groups

	Groups				
	Control	EGCG	CDDP	CDDP+EGCG	*P*
Urea nitrogen (mg/dL)	43.0 ± 6.2[c]	37.5 ± 6.8[c]	135.3 ± 14.1[a]	91.6 ± 8.2[b]	.0001
Creatinine (mg/dL)	0.42 ± 0.06[c]	0.41 ± 0.06[c]	1.62 ± 0.19[a]	0.70 ± 0.12[b]	.0001
MDA (nmol/g)	90.5 ± 30.5[c]	84.6 ± 26.3[c]	179.1 ± 93.3[a]	108.0 ± 38.2[b]	.0001

[a, b, c]Differences between the groups marked with different letters are statistically significant ($P < .05$).
MDA, malondialdehyde.

identified. Using the mouse monoclonal anti-β-actin antibody, protein binding was determined (A5316; Sigma). Protein levels were densitometrically measured using an image recognition method (Image J; National Institute of Health, Bethesda, USA).

Histopathology

Left kidneys obtained from each rat were rapidly fixed in 20% neutral buffered formalin solution. They were then eventually dehydrated and paraffin-embedded. Paraffin blocks were sliced according to the standard procedures at a thickness of 5 mm and stained with hematoxylin-eosin.[13] For each kidney slide, a minimum of 10 areas have been assessed. A pathologist who was blind to treatment classes measured semi-quantitatively the existence of vacuolar degeneration, tubular atrophy and dilation, tubular necrosis, interstitial edema, and inflammation. Using the following rating scale, the magnitude of change was expressed as follows: (–): none, (+): slight change, (++): moderate change, (+++): significant change.

Statistical Analysis

All experimental data were presented in the form of mean ± SEM. Data were analyzed using the PROC GLM (Generalized Linear Model) procedure in SAS (Statistical Analysis Systems, SAS Institute Inc., Cary, NC, USA, 2002) software package. The groups were compared using one-way variance analysis (ANOVA) followed by the Fisher post hoc test. Statistical significance was accepted as $P < .05$.

RESULTS

Renal Function

The CCDP group was found to have significantly higher urea and creatinine levels (141.8 mg/dL, 1.51 ± 0.12 mg/dL) compared to the control group (41.0 mg/dL, 0.43 mg/dL) ($P < .0001$). However, urea and creatinine levels of the group treated with CDDP + EGCG (84.8 mg/dL, 0.61 mg/dL) were significantly lower than those of the group treated with CDDP alone (141.8 mg/dL, 1.51 ± 0.12 mg/dL) ($P < .0001$). The differences between the groups are summarized in Table 1.

Serum MDA Levels

Cross-group differences in levels of MDA, which is a factor of oxidative stress, were found to be significant ($P < .001$). Experimental groups can be listed in descending order of serum MDA levels as follows: CDDP group > CDDP + EGCG group > control group > EGCG group. The CDDP group was determined to have significantly higher MDA levels (179.1 ± 93.3 nmol/g) than the control group (90.5 ± 30.5 nmol/g) and other groups ($P < .005$). The EGCG group was determined to have significantly lower MDA levels compared to the control group ($P < .005$). The EGCG (84.6 ± 26.3 nmol/g) and CDDP + EGCG (108.0 ± 38.2 nmol/g) groups, which received EGCG treatment, were detected to have significantly lower serum MDA levels than the CDDP group (179.1 ± 93.3 nmol/g) ($P < .05$) (Table 1).

Renal Bax, Bcl-2, Hsp70 Expression

In the CDDP group, the Bax expression was greater than in the control group ($P = .05$). The CDDP + EGCG group showed slightly lower Bax expression relative to the CDDP group ($P = .05$) (Figure 1A). When Bcl-2 levels were compared across the EGCG and control groups, no statistically significant differences were found. In the CDDP + EGCG group, the levels of Bcl-2, assumed to play a protective role in apoptosis, were substantially higher than in the other groups (Figure 1B). The groups can be listed in descending order of Hsp 60 expression levels, which are thought to increase during tubular damage, as follows: CDDP > CDDP + EGCG > control > EGCG. The CDDP group was determined to have significantly higher Hsp60 expression than all of the other groups ($P < .05$). The CDDP + EGCG group displayed a slightly decreased Hsp60 expression ($P < .05$) relative to the CDDP group (Figure 1C). In the CDDP group, Hsp70 expression levels were substantially higher compared with all the other groups ($P < .001$). The CDDP + EGCG group showed substantially diminished expression of Hsp70 relative to the CDDP group ($P < .05$) (Figure 1D).

Histopathological Results

No pathologies were detected in kidney preparations obtained from the rats in the control and EGCG groups. However, the group treated with CDDP demonstrated vacuolization in the cortex and the outer medulla, interstitial edema and inflammation, and severe tubular necrosis and tubular atrophy. Meanwhile, the EGCG + CDDP group treated with EGCG was observed to demonstrate quite fewer histopathological changes induced by CDDP (esp. Tubular necrosis and interstitial inflammation) (Table 2, Figure 2).

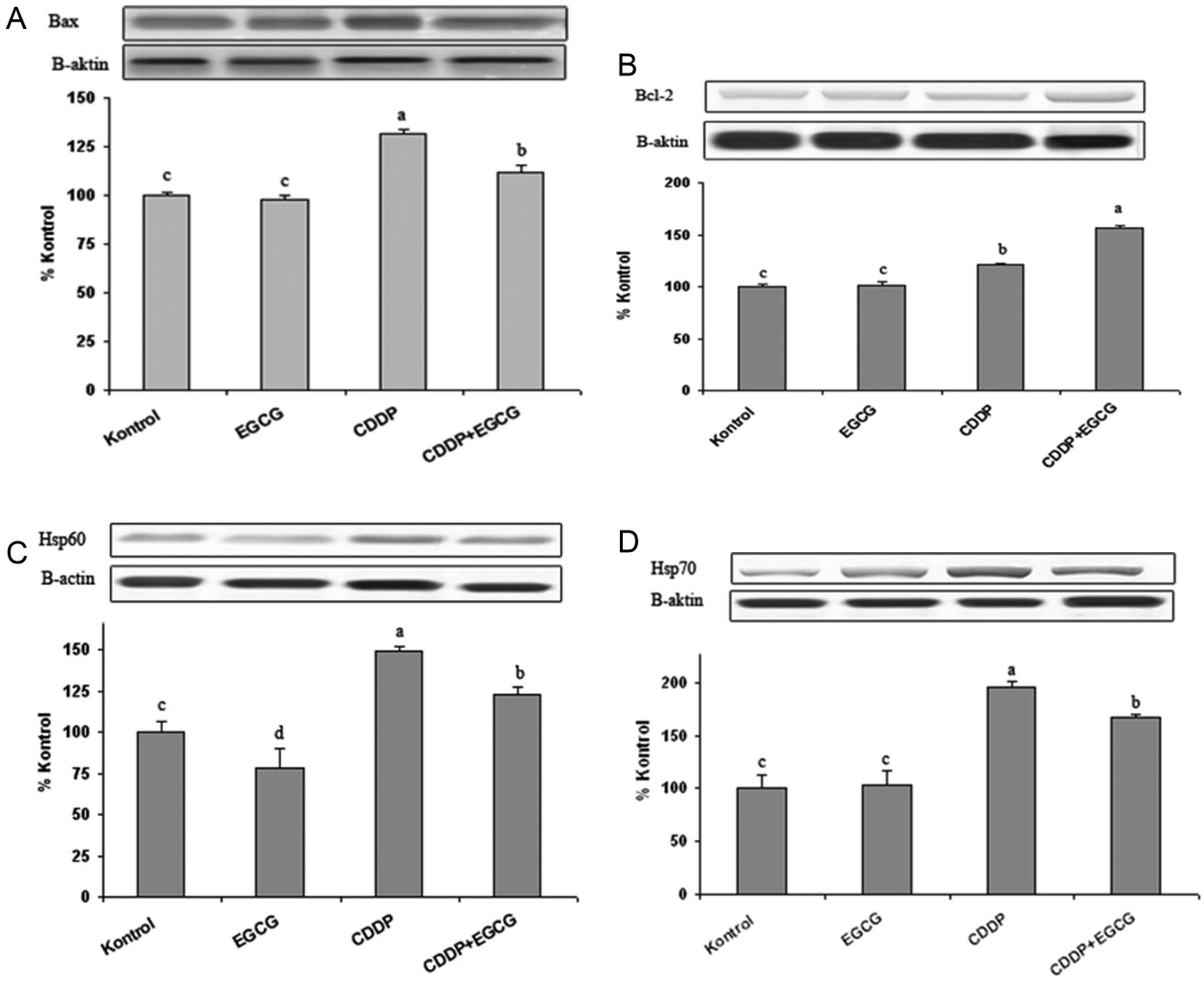

Figure 1. a-d. Effect of EGCG on Bax (A), B-cell lymphoma-2 (Bcl-2) (B), heat shock protein 60 (Hsp60) (C) and heat shock protein 70 (Hsp70), and (D) expressions in rat kidney model treated by cisplatin. By densitometric study, the amplitude of the bands was detected. The bar reflects the average standard error. To insure equivalent protein loading, actin was used. Data points with different superscripts are significantly different from the Fisher multiple comparison test at the level of $P < .05$.

DISCUSSION

In cytotoxic chemotherapies, nephrotoxicity is a common side effect that affects tolerance and dosage restriction. There are many cellular targets in CDDP nephrotoxicity, and they can be grouped into 5 main sections: DNA damage; cytoplasmic organelle dysfunction, with endoplasmic reticulum stress and mitochondrial dysfunction; apoptotic pathways, both caspase-dependent and death receptor-mediated; oxidative stress, with the formation of ROS; and inflammation. The failure to remove free oxygen radicals that are produced as a result of reactions that occur after CDDP enters the kidney proximal tubule cell causes oxidative stress in CDDP nephrotoxicity.[14] Cellular antioxidant systems can be divided into 2 major groups: non-enzymatic and enzymatic. Non-enzymatic defense includes low molecular weight of antioxidant compounds such as vitamins C and E, different selenium compounds, GSH, lipoic acid, and ubiquinone. The enzymatic defense comprises agents that catalytically remove ROS; for instance, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase, and glutathione-S-transferase.[15] Therefore, studies have investigated

Table 2. Impact of the Application of EGCG on Morphological Changes in the Tissue of Rat Kidneys

	Groups			
Morphological Changes	**Control**	**EGCG**	**CDDP**	**CDDP+EGCG**
Vacuolization	–	–	++	+
Interstitial edema	–	–	+	–
Tubular necrosis	+/–	+/–	+++	+
Tubular atrophy	–	–	++	+/–
Interstitial inflammation	–	–	++/+++	+

–, none; +, slight change; ++, moderate change; +++, significant change.

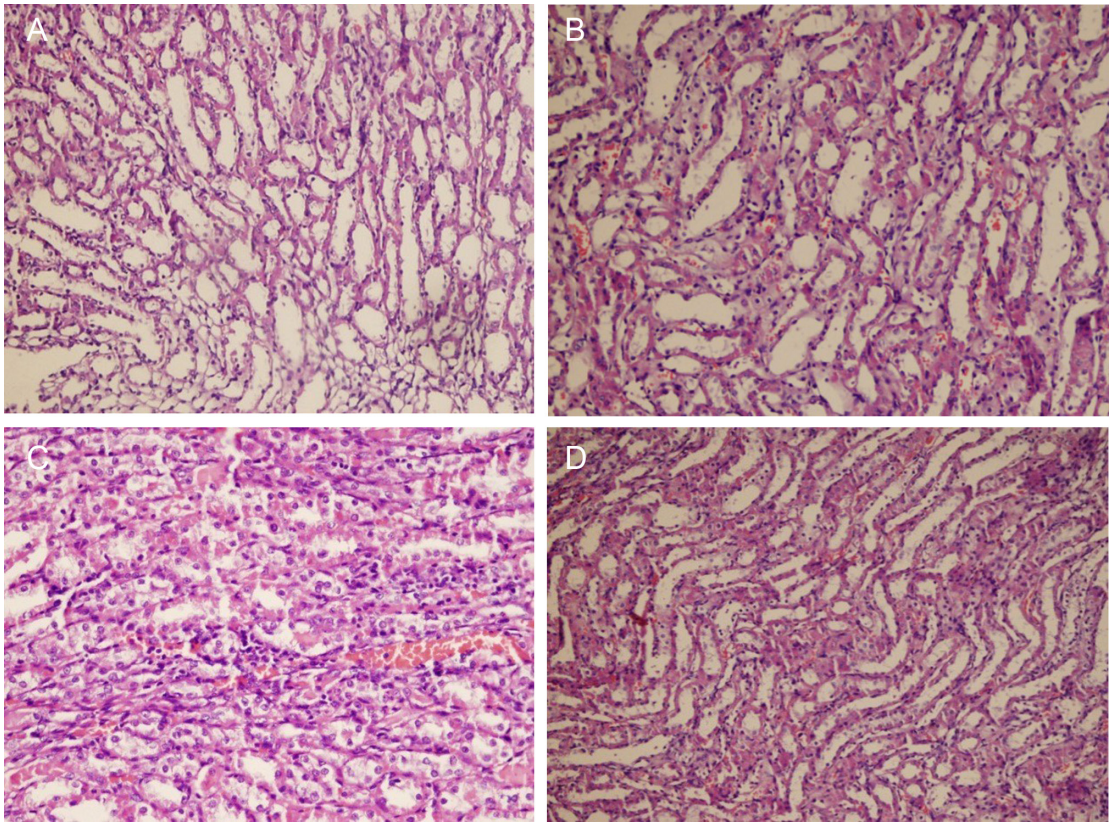

Figure 2. a-d. Histopathological appearance of the kidney in different groups, stained with hematoxylin-eosin (H&E). Control (A), EGCG (B), CDDP (C), and CDDP + EGCG (D).

the preventive roles of certain antioxidant substances such as ginkgo alkaloids, vitamin C, acetylsalicylic acid, ebselen, curcumin, taurine, misoprostol, bixin, lipoic acid, SOD, selenium, flavonoids, diethyldithiocarbamates, erdosteine, caffeic acid phenethyl ester, and Nigella sativa extract in experimental animals treated with CDDP.[16–19] One of the mechanisms of action of EGCG, which is found in green tea and was used in the present study, that has been studied most extensively is its antioxidant property. EGCG was shown to dramatically decrease plasma levels of both lipid and DNA oxidative damage biomarkers.[20] EGCG can also prevent cellular oxidative damage by inhibiting lipoxygenase, cyclooxygenase, and xanthine-oxidase, which can potentially create oxidative damage in certain tissues through peroxidative activity.[21] As a marker of oxidative stress, serum MDA levels were used in our research. MDA is one of the most powerful and well-accepted indicators of lipid peroxidation in vivo.[22] In rat studies conducted by Li and Xie,[23] phenolic substances in tea were found to prevent damage to and the oxidation of the SOD enzyme, which breaks down dangerous radical species, thus increasing the SOD enzyme activity and reducing the amount of MDA, a product of lipid oxidation. In another study, green tea catechins were shown to be efficient in lowering oxidative stress by reducing the activity of the SOD enzyme in the liver and increasing the activity of the CAT enzyme.[24] Bolaman et al.[25] treated the CDDP group with 10 mg/kg of CDDP and treated the placebo group with 200 mg/kg of vitamin E at the same time as the CDDP treatment in a rat study to examine the role of alpha-tocopherol in preventing CDDP-induced lipid peroxidation in the rat kidney. They concluded that the CDDP group's renal tissue MDA levels were considerably higher than those of the controls, suggesting increased lipid peroxidation in the CDDP group. The group treated with alpha-tocopherol reported slightly lower levels of MDA than the group treated with CDDP, and the authors indicated that alpha-tocopherol could be used to inhibit lipid peroxidation caused by CDDP. In line with these results, the current study detected substantially higher levels of serum MDA in the CDDP-treated group relative to the control group. However, adding EGCG to CDDP resulted in a decrease in MDA levels. These findings show that the use of EGCG restores the antioxidant defense system in the serum.

Apoptosis is regulated by numerous accessory genes. These genes either promote programmed cell death (p53, Bax, c-myc) or inhibit it (Bcl-2, Bcl-xl, setrin).[26] CDDP nephrotoxicity was observed to be accompanied by inflammation and apoptosis. Increases in the pro-apoptotic Bax protein and decreases in the anti-apoptotic Bcl protein in CDDP nephrotoxicity were seen by Francescato et al.[27] Similarly, another study demonstrated that the acute kidney injury in CDDP nephrotoxicity was

accompanied by the activation and accumulation of the Bax gene in renal tubular cell mitochondria.[28] Following ischemic-reperfusion damage, elevated Bcl-2 in tubular epithelial cells can suppress cellular autophagy and inhibit apoptosis.[29] Our analysis, therefore, determined that in the CDDP group, Bax expression was substantially higher than in the control group. The CDDP + EGCG group showed substantially decreased Bax expression relative to the CDDP group. In the CDDP + EGCG group, levels of Bcl-2, which is thought to play a protective role in apoptosis, were substantially higher relative to the other groups.

Recent studies have demonstrated a strong correlation between Hsps and oxidative stress.[30] Takayuki et al.[31] showed that dimethylthiourea (DMTU), which is a strong antioxidant, prevents Bax-induced apoptosis by suppressing Hsp60 in renal tubular cells in the early period and thus plays a protective role against acute renal failure caused by CDDP. As with DMTU, the protective effect against oxidative stress was revealed to arise from the suppression of Hsp expression in various in vitro studies.[32] Our findings suggest that Hsp60 and Hsp70 levels of the EGCG + CDDP group treated with EGCG, although not as low as baseline levels (levels of the control group), are lower than those of the CDDP group, and that the protective effect against CDDP nephrotoxicity is at least partially due to Hsps. In addition, these results corroborate the previous findings suggesting that there is a notable positive correlation between EGCG use and Hsp expression in rats treated with CDDP, and that Hsp expression is altered, and the tissues are more susceptible to damage in rats treated with CDDP.

In the development of CDDP-induced nephrotoxicity, decreased creatinine clearance is among the early symptoms. In the present study, these parameters of poor renal function were found to be significantly higher in the CDDP group compared to the control and EGCG groups. In the group treated with EGCG in addition to CCDP, the EGCG treatment was observed to counter the expected CDDP-induced increase in urea and creatinine levels. This result shows that EGCG improves renal function. Mansour et al.[33] reported that serum urea and creatinine levels increased 2.5 to 3.3 times, respectively, in the nephrotoxicity group they created with 7.5 mg/kg CDDP compared to the control group. They reported that although serum urea and creatinine levels were higher than the control group in the group treated with amyguanidine for 5 days before CDDP, they decreased significantly compared to the CDDP group. Durak et al.[34] administered vitamin E + C complex and the natural antioxidant Sarmex to guinea pigs they had treated with CDDP in order to observe their effects on the development of neurotoxicity. They reached the conclusion that CDDP had negative effects on both the enzymatic and non-enzymatic antioxidant systems, and that the antioxidants they used prevented kidney failure that develops secondary to CDDP. In conclusion, various agents with nephroprotective effects that have been investigated in our study and others in the literature were shown to induce a significant decrease in urea and creatinine levels, which was directly proportional to a decrease in lipid peroxidation.[33,34] This result that was obtained in our study through biochemical methods was also supported by the histopathological results. Findings that result from CDDP, such as tubular necrosis, interstitial inflammation, and vacuolization, were revealed to be prevented at a significant scale with EGCG treatment.

In conclusion, in rats treated with CDDP, complementing the treatment with EGCG was found to result in a down-regulation of the Bax protein and Hsp60-70. These data, as supported by biochemical and histopathological findings, show that the renal damage caused by CDDP is attenuated by EGCG, which is an antioxidant. This effect can be linked to a decrease in the levels of oxidative stress markers and heat shock proteins.

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