Archives of Medical Research

Archives of Medical Research 46 (2015) 517−526

ORIGINAL ARTICLE

Protective Effects of Apocynin on Cisplatin-induced Hepatotoxicity in Rats

Yasir Furkan Cagin,[a] Mehmet Ali Erdogan,[a] Nurhan Sahin,[b] Hakan Parlakpinar,[c] Yahya Atayan,[a] Alaadin Polat,[d] Nigar Vardi,[e] Azibe Yildiz,[e] and Kevser Tanbek[d]

[a]*Department of Gastroenterology,* [b]*Department of Pathology,* [c]*Department of Pharmacology,* [d]*Department of Physiology,* [e]*Department of Histology and Embryology, Medical Faculty, Inonu University, Malatya, Turkey*

Received for publication February 28, 2015; accepted August 20, 2015 (ARCMED-D-15-00134).

Background and Aims. Despite it being a highly potent antineoplastic drug, cisplatin has important toxic adverse effects limiting its use such as nephrotoxicity, neurotoxicity and ototoxicity. It is thought that cisplatin-induced hepatotoxicity is caused by oxidative stress resulting from increased reactive oxygen species (ROS). Apocynin (APO) exerts its antioxidant effect by reducing ROS production via inhibition of NADPH oxidase. The present study intended to demonstrate effects of cisplatin on hepatic pro-oxidant/antioxidant systems and to investigate protective effects of APO against cisplatin-induced hepatotoxicity.

Methods. Rats were randomly assigned into four groups ($n = 8$ each): a) control group; b) single dose of cisplatin (5 mg/kg); c) APO group (20 mg/kg on three consecutive days; i.p.); and d) APO plus cisplatin group. Liver tissue was assessed in all groups by biochemical and histopathological means. Also, serum alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase levels were studied in all groups.

Results. When cisplatin group was compared to controls, it was seen that lipid peroxidation product, total oxidant status and ALT levels were markedly increased, whereas superoxide dismutase and glutathione peroxidase levels were overtly decreased. APO therapy markedly prevented cisplatin-induced harmful changes in liver. Our histopathological findings such as central vein dilatation, perivenuler and periportal sinusoidal dilatation, parenchymal inflammation, vacuolar changes in hepatocytes, biliary duct proliferation and caspase-3 positive hepatocytes were in accordance with the biochemical changes.

Conclusion. In light of these results, it is our thought that APO has a protective role against cisplatin-induced hepatotoxicity at both biochemical and histopathological levels. © 2015 IMSS. Published by Elsevier Inc.

Key Words: Cisplatin, Apocynin, Hepatotoxicity, Oxidative stress.

Introduction

Cisplatin (cis-diamminedichloroplatinum [II]) is a potent antineoplastic agent commonly used in many cancers including testis, ovary, bladder, lung, kidney and head-neck cancers (1−3). It is an organic platinum derivative in contrast to other antineoplastic agents (4,5). Despite its potent anti-tumoral effect, it has important toxic side effects such as nephrotoxicity, neurotoxicity and ototoxicity, which limits its clinical use (6−9). In addition, hepatotoxicity also develops due to accumulation of its metabolites (10,11). It was shown that cisplatin causes hepatotoxicity development at higher doses although hepatotoxicity is not considered as a dose-limiting toxic effect (12,13). However, hepatotoxicity is also encountered during low-dose repeated cisplatin therapy (14).

In recent studies it has been reported that the mechanism in cisplatin-induced hepatotoxicity can be multifactorial. Oxidative stress resulting from ROS may have a major role among these factors (15,16). In addition to the oxidative

Address reprint requests to: Yasir Furkan Cagin, MD, Department of Gastroenterology, Faculty of Medicine, Inonu University, 44280, Malatya, Turkey; Phone: (+90) (422) 341 0660-4112; FAX: (+90) (422) 341 0036; E-mail: yasir.cagin@inonu.edu.tr or yafur@hotmail.com

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http://dx.doi.org/10.1016/j.arcmed.2015.08.005

stress, apoptosis also plays an important role in cisplatin-induced hepatotoxicity (17). These experimental studies investigate several antioxidant substances in cisplatin-induced hepatotoxicity. Because it is assumed that cisplatin-related adverse effects can occur through various ways, the idea that different chemical agents that may decrease the potential adverse effects can be used in combination seems to be reasonable. In the present study we investigated the protective effect of apocynin, a potent free radical scavenger and antioxidant, against cisplatin-induced hepatotoxicity.

Apocynin (APO) is a phenolic compound obtained originally from the Picrorhiza kurroa plant belonging to apocynum species of North America and Asia (18,19). APO is an inhibitor of NADPH-oxidase (NOX), which was proven in several cell cultures and animal studies (18,19). It was shown that apocynin is a pro-oxidant enzyme reducing oxygen molecule and oxidizing NADPH through this activity (20,21). It is an enzyme responsible for ROS formation. Excessive ROS formation leads to clinical disorders by increasing oxidative stress and apoptotic cell death. APO exerts its antioxidant effect by decreasing ROS via NOX inhibition (22,23). More recently, Kilic et al. reported that APO can diminish neutrophil oxidative burst and neutrophil chemotaxis, and thus reduce neutrophil-mediated cell injury (24). The authors showed protective and therapeutic effects of APO against bleomycin-induced lung fibrosis. Also, more recently Ozbek et al. reported that APO exerts beneficial effects against testicular ischemia-reperfusion injury (25). In the light of above mentioned investigations it may be suggested that APO has become the focus of interest for the treatment of some diseases in several studies.

Therefore, in the current study, we focused on evaluating the protective effects of APO against cisplatin-induced hepatic injury in rats at both histopathological and biochemical levels.

Materials and Methods

Animals

The study was approved by the Inonu University Ethics Committee on Experimental Animal Research (Reference number: 2014/A-36). Thirty two female Wistar Albino rats (there was no specific reason for using female rats) aged 4 months (weighing 200−230 g) were used in the study. Rats were supplied by the Inonu University Experimental Animal Research Center. All animals were fed with standard rat pellet and tap water with 24 h fasting before experiment. All rats were housed in appropriately ventilated rooms under standard conditions with temperature of 21 ± 2°C and humidity of 60 ± 5% by maintaining a 12 h dark-light cycle. Experimental groups were formed by assigning rats randomly. The groups were housed separately throughout the experiment.

Experimental Protocol

Rats were randomly assigned to four groups ($n = 8$ each): Group I—control group injected with the same volume; Group II—single-dose cisplatin (5 mg/kg; Cisplatin DBL; 50 mg Orna Corp., Istanbul, Turkey) was given via intraperitoneal (i.p.) route; Group III—APO (20 mg/kg) was given i.p. over three consecutive days; Group IV—injected with a single dose of cisplatin (5 mg/kg, i.p.) plus APO (20 mg/kg, i.p.) 1 h before cisplatin-treatment and continued during the other 2 days to test the protective effect of APO against cisplatin-induced hepatotoxicity.

All rats were sacrificed by an overdose of ketamine (100 mg/kg, i.p; Ketolar; Parke-Davis, Spain) and xylazine (10 mg/kg, i.p; Alfazyne 2%; Alfasan, The Netherlands) mixture on day 4 of the experiment. After sacrifice, liver tissue specimens were harvested quickly and meticulously for biochemical and histopathological analyses.

Liver tissue was placed in liquid nitrogen and stored at −70°C until assayed for malondialdehyde (MDA), a lipid peroxidation product, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione (GSH), total antioxidant capacity (TAC), total oxidant status (TOS) and oxidative stress index (OSI) contents. The other portion of the liver was placed in formaldehyde solution for routine histopathological examination by light microscopy. Trunk blood was extracted to evaluate the serum levels of alanine transaminase (AST), aspartate transaminase (AST) and alkaline phosphatase (ALP) using an Olympus Autoanalyzer (Olympus Instruments, Tokyo, Japan).

Doses of cisplatin and APO were chosen as dependent on the previous dose-response studies that have been reported to cause hepatotoxicity (3,14,26) and marked antioxidative effects in rats (27), respectively.

Histopathological Methods

For light microscopic analysis, liver samples were fixed in 10% formalin for 48 h, dehydrated in ascending alcohol series and embedded in paraffin. Paraffin blocks were prepared for sectioning at 4 μm thickness by microtome. The sections were stained with hematoxylin and eosin (H-E).

For immunohistochemical analysis, the sections were mounted on polylysine-coated slides. After deparaffinization and rehydration procedures, sections were placed in antigen retrieval solution (citrate buffer, pH 6.0) and boiled in a pressure cooker for 20 min and cooled to room temperature for 20 min. The sections were then washed with phosphate-buffered saline (PBS). After washing the sections, 3% hydrogen peroxide solution was applied to block endogenous peroxide for 15 min at room temperature and washed with PBS. Protein block was applied to the

sections. The sections were incubated with primary rabbit-polyclonal caspase-3 antibody (Neomarker, USA) for 60 min, rinsed in PBS and incubated with biotinylated goat antipolyvalent for 20 min and streptavidin peroxidase for 20 min at room temperature. Staining was completed with chromogen + substrate for 10 min, and slides were counter-stained with Mayer's hematoxylin for 1 min, rinsed in tap water, and dehydrated. Caspase-3 kit was used according to the manufacturer's instructions.

Tissue preparations were stained by hematoxylin and eosin (HE) and evaluated by a pathologist blinded to groups under light microscope (Nikon Eclipse, E600 W. Tokyo, Japan). Pictures were captured using a digital camera (Nikon Microscope Digital Camera DP70, Tokyo, Japan). In histopathological evaluation, the following findings were assessed as positive or negative:

- Central vein dilation
- Central vein thrombosis
- Central vein congestion
- Perivenuler sinusoidal dilation
- Periportal sinusoidal dilation
- Parenchymal inflammation
- Vacuolar changes in hepatocytes
- Bile duct proliferation

Full form of caspase-3 antibody was used to detect caspase-3 in IHC. Caspase-3 positive cells stained as brown color. Stained hepatocytes with caspase-3 were counted by examining 20 areas for each specimen (as 62.5 μm × 20 μm) under a 20X objective. All sections were evaluated using a Leica DFC280 light microscope and a Leica Q Win Image Analysis system (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

Biochemical Analysis

Two hundred milligrams of frozen liver tissue was cut into pieces on dry ice and homogenized in 10 vol of ice-cold Tris-HCl buffer with respect to tissue weight (50 mmol/L, pH 7.4) using a homogenizer (Ultra Turrax IKAT18 basic homogenization; Werke, Staufen, Germany) for 3 min at 6000 rpm. The supernatant solution was extracted with an equal volume of an ethanol/chloroform mixture (3/5, volume per volume [v/v]). After centrifugation at 3000 g for 30 min, the upper layer was used in the analysis of total tissue protein levels.

Determination of MDA

MDA contents of the homogenates were determined spectrophotometrically by measuring the presence of thiobarbituric acid reactive substances (TBARS) (28). Three mL of 1% phosphoric acid and 1 mL 0.6% thiobarbituric acid solution were added to 0.5 mL of homogenate pipetted into a tube. The mixture was heated in boiling water for 45 min. After the mixture cooled, the colored part was extracted into 4 mL of n-butanol. Absorbance was measured by spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan) at 532 and 520 nm. The amount of lipid peroxides was calculated as TBARS of lipid peroxidation. Results were expressed in nmol/g tissue according to a standard graph, which was prepared using the measurements of standard solutions (1, 1, 3, 3-tetramethoxypropane).

Determination of Protein Content

Protein content of the samples was determined by the method of Lowry et al. (29) using bovine serum albumin as a standard.

Determination of SOD Activity

Total SOD activity was determined based to the method of Sun et al (30). The principle of the method is the inhibition of nitrobluetetrazolium (NBT) reduction by the xanthine-xanthine oxidase system as a superoxide generator. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. SOD activity was calculated as units per milligram protein (U/mg protein).

Determination of CAT Activity

CAT activity was determined according to Aebi's method (31). The principle of the assay is based on the determination of the rate constant (k, s-1) or the H_2O_2 decomposition rate at 240 nm. Results are expressed as k per gram protein (k/g protein).

Determination of GPx Activity

Determination of GPx activity was measured by the method of Paglia and Valentine (32). An enzymatic reaction in a tube containing NADPH, GSH, sodium azide, and glutathione reductase was initiated by addition of H_2O_2, and the change in absorbance at 340 nm was observed by a spectrophotometer. Activity was given in units per gram protein (U/g protein).

Determination of GSH Content

GSH concentration in homogenate was measured spectrophotometrically according to the method of Ellman. GSH content in liver tissue as non-protein sulfhydryl was analyzed with this described method (33). Aliquots of tissue homogenate were mixed with distilled water and 50% trichloroacetic acid in glass tubes and centrifuged at 3000 rpm for 15 min. The supernatants were mixed with Tris buffer (0.4 mol, pH 8.9) and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, 0.01 mol) was added. After shaking the reaction mixture, its absorbance was measured at 412 nm within 5 min of the addition of DTNB against blank with no homogenate. The absorbance values were extrapolated

from a glutathione standard curve and expressed as GSH (μmol/g tissue).

Determination of TAC

TAC levels were determined using a novel automated colorimetric measurement method developed by Erel (34). In this method, the hydroxyl radical, the most potent biological radical, is produced by the Fenton reaction and reacts with the colorless substrate O-dianisidine to produce the dianisyl radical, which is bright yellowish-brown in color. Upon the addition of the sample, the oxidative reactions initiated by the hydroxyl radicals present in the reaction mix are suppressed by the antioxidant components of the sample, preventing the color change and thereby providing an effective measure of the total antioxidant capacity of the sample. The assay has excellent precision values, which are $<3\%$. Results were expressed as mmol Trolox equivalent/L.

Determination of TOS

TOS was determined using a novel automated measurement method developed by Erel (34). The oxidants present in the sample oxidize the ferrous ion–O-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric iron results in a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of the oxidant molecules present in the sample. The assay was calibrated with hydrogen peroxide and the results were expressed in terms of μmol H_2O_2 equivalent/L.

Measurement of OSI

The ratio of the TOS to TAC was accepted as the OSI, an indicator of the degree of oxidative stress (34). The OSI value was calculated from the formula:

OSI (arbitrary unit) = TOS/TAC.

The OSI value of the liver samples was also calculated as an OSI (arbitrary unit).

Statistical Analysis

Data were expressed as either median (min-max) values or mean ± standard deviation (SD) depending upon the overall variable distribution. Normality was assessed using Shapiro-Wilk test. Normally distributed data were analyzed by one-way ANOVA followed by Tukey post-hoc test. Non-normally distributed data were compared by Kruskal Wallis *H* test among the groups. When significant differences were determined, multiple comparisons were carried out using the Mann-Whitney U test with Bonfernoni correction; $p < 0.05$ was considered as significant. IBM SPSS statistics v.22.0 for Windows was used for statistical analyses.

Results

Body and Liver Weights

None of the animals died during the experimental period. There were no significant differences in the body and liver weights obtained before and after the experiments among the groups (data not shown).

Biochemical Results

The results of the biochemical parameters of the pro- and antioxidant systems belonging to the liver tissue are presented in Table 1. Briefly, MDA levels were found to be significantly higher in the cisplatin group when compared to the control group ($p < 0.001$). MDA levels were found to be much lower in the cisplatin + APO group than the cisplatin group, which is statistically significant ($p < 0.05$) (Table 1). When SOD, CAT, GPx and GSH levels were compared between the cisplatin and control groups, it was observed that there were marked differences in all parameters (Tables 1 and 2). However, statistically significant differences were observed in SOD and GPx levels ($p < 0.001$ and $p < 0.01$, respectively) (Tables 1 and 2). When the same parameters were compared between the cisplatin and the cisplatin + APO groups, significant improvements were observed in all parameters ($p < 0.01$, $p < 0.05$, $p < 0.001$ and $p < 0.01$, respectively) (Tables 1

Table 1. Comparison of the tissue biochemical parameters among the study groups

Groups ($n = 8$)	MDA (nmol/g tissue) median (min–max)	SOD (U/mg protein) median (min–max)	CAT (k/g protein) median (min–max)	GPx (U/g protein) median (min–max)
Control	10.30 (7.26–12.21)	2.00 (1.80–2.78)	29.90 (24.29–55.02)	218.09 (167.35–293.75)
Cisplatin	22.78 (11.42–39.38)[a]	1.12 (1.02–1.42)[a]	25.03 (9.45–31.19)	155.42 (103.53–166.34)[a]
APO	12.80 (8.45–16.55)	1.73 (1.61–2.69)[b]	35.23 (22.57–55.98)	190.10 (159.38–271.35)[b]
Cisplatin + APO	11.49 (8.62–15.67)[b]	2.11 (1.65–2.55)[b]	36.03 (28.35–46.258)[b]	217.55 (179.94–279.98)[b]
p	<0.001	<0.001	<0.05	<0.001

APO, apocynin; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.
Results are expressed as median (min–max), $n = 8$.
[a] $p < 0.05$ vs. control group.
[b] $p < 0.05$ vs. cisplatin group.

Table 2. Comparison of the tissue biochemical parameters among the study groups

Groups (n = 8)	GSH (μmol/g tissue) median (min−max)	TOS (μmol/g tissue) median (min−max)	TAC (mmol trolox Eq/L) median (min−max)	OSI (arbitrary unit) median (min−max)
Control	9.20 (7.84−10.36)	24.41 (16.09−28.05)	1.51 (1.31−1.62)	17.06 (10.16−19.11)
Cisplatin	7.84 (5.55−8.93)	36.69 (27.50−47.82)[a]	1.47 (1.33−1.73)	23.74 (19.69−33.53)[a]
APO	8.91 (7.19−10.84)	27.68 (19.40−32.65)	1.54 (1.39−1.68)	18.52 (12.64−20.79)
Cisplatin + APO	10.02 (7.36−11.74)[b]	24.37 (16.09−31.91)[b]	1.58 (1.48−1.66)	15.28 (10.16−20.49)[b]
p	<0.05	<0.001	>0.05	<0.001

APO, apocynin; GSH, reduced glutathione; TOS, total oxidative stress; TAC, total antioxidant capacity; OSI, oxidative stress index.
Results are expressed as median (min−max), n = 8.
[a] $p < 0.05$ vs. control group.
[b] $p < 0.05$ vs. cisplatin group.

and 2). When TOS, TAC and OSI levels were compared between the cisplatin and the control groups, it was found that there were significant differences in all parameters other than TAC ($p < 0.01$ and $p < 0.01$) (Tables 1 and 2). When the same parameters were compared between the cisplatin and the cisplatin + APO groups, it was observed that there were significant differences in all parameters other than the TAC ($p < 0.01$ and $p < 0.01$) (Tables 1 and 2).

When ALT, AST and ALP levels were compared between cisplatin and control groups, significant differences were found in ALT and ALP levels ($p < 0.01$ and $p < 0.01$, respectively) (Table 3). When the same parameters were compared between cisplatin and cisplatin + APO, the only significant difference was observed in ALT level ($p < 0.05$) (Table 3).

Histopathological Results

Histopathological findings are presented in Table 4 and Figure 1. In brief, microscopically, no histopathological change was observed in the control and APO groups (Figures 1A and F). In the cisplatin group there was central vein dilatation, vacuolar changes in hepatocytes, central vein thrombosis, central venous congestion, perivenular sinusoidal dilatation, periportal sinusoidal dilatation and parenchymal inflammation (Figures 1B−E). Parenchymal inflammation and central vein thrombosis were observed in the cisplatin + APO group (Figures 1G and H). However, in the cisplatin + APO group it was observed that the central venous congestion perivenular sinusoidal dilatation and central vein thrombosis were decreased.

No apoptotic cells were observed in the caspase-3 study in the control and APO groups except for a few cells (Figures 2A and C). However, caspase-3 positive cells were found to be increased in the cisplatin group when compared to the control group (Figure 2B) ($p < 0.05$). On the other hand, the number of the caspase-3 positive cells was decreased by administration of APO (Figure 2D) ($p < 0.05$). The number of the apoptotic cells for each group is given in Table 4.

Discussion

Cisplatin is one of the potent anti-neoplastic agents commonly used in cancer therapy (35). Although there are many experimental studies on common toxic effects associated with cisplatin when used in high doses, the number of studies about hepatotoxicity is still insufficient (36). The mechanisms underlying the cisplatin-induced hepatotoxicity have not yet been fully elucidated. However, there are studies indicating that oxidative stress may play a major role. Excessive production of ROS causes apoptosis as well as oxidative stress. Thus, apoptosis also plays an important role in hepatotoxicity (16,37). In previous studies, it was shown that the NADPH-oxidase is the main source of ROS (38). It was shown that ROS are increased by cisplatin use, leading to

Table 3. Effects of apocynin on serum parameters in rats treated with cisplatin

Groups (n = 8)	ALT (IU/L) median (min−max)	AST (IU/L) median (min−max)	ALP (IU/L) median (min−max)
Control	36.50 (27.00−46.00)	98.00 (64.00−118.00)	71.00 (42.00−145.00)
Cisplatin	64.50 (52.00−83.00)[a]	132.00 (98.00−243.00)	141.00 (62.00−346.00)[a]
APO	47.50 (31.00−102.00)	131.00 (78.00−218.00)	125.00 (81.00−234.00)[a]
Cisplatin + APO	40.50 (30.00−64.00)[b]	111.50 (71.00−187.00)	107.50 (53.00−118.00)
p	<0.01	>0.05	<0.01

APO, apocynin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase.
Results are expressed as median (min−max), n = 8.
[a] $p < 0.05$ vs. control group.
[b] $p < 0.05$ vs. cisplatin group.

Table 4. Histopathological findings

Pathological changes	Control ($n = 8$)	Cisplatin ($n = 8$) %	APO ($n = 8$) %	Cisplatin + APO ($n = 8$) %
Central vein dilation	-	50 (4/8)	-	37.5 (3/8)
Vacuole changes in hepatocytes	-	12.5 (1/8)	-	-
Bile duct proliferation	-	-	-	-
Central vein thrombosis	-	25 (2/8)	-	12.5 (1/8)
Central vein congestion	-	37.5 (3/8)	-	25 (2/8)
Perivenuler sinusoidal dilation	-	50 (4/8)	-	25 (2/8)
Periportal sinusoidal dilation	-	12.5 (1/8)	-	-
Parenchymal inflammation	-	12.5 (1/8)	-	-
Caspase-3 positive cells	0.0 (0.0–2.0)	0.0 (0.0–6.0)[a]	0.0 (0.0–1.0)	0.0 (0.0–2.0)[b]

APO, apocynin.
[a]Statistically significant increase when compared with control group ($p = 0.001$).
[b]Statistically significant decrease when compared with cisplatin group ($p = 0.001$).

DNA and protein damage and lipid membrane peroxidation (39). Thus, experimental studies were conducted, which aimed to decrease or prevent the hepatotoxic effects of cisplatin by using some substances with known antioxidant effects (40,41). We planned this study in order to test the protective effect of APO, a powerful antioxidant and free radical scavenger agent, against cisplatin-induced hepatotoxicity. This study differs from those in the literature with it being the first study. Many authors demonstrated that APO decreases inflammation, ROS and/or oxidative stress and has beneficial effects on the cells (19,42).

In the current study, a single dose of cisplatin (5 mg/kg) induced hepatotoxicity. This situation was shown by histopathological changes, with significant increase of the hepatic oxidative stress markers and serum ALT level. It is thought that oxidative stress is the most important cause of the hepatic changes related to the cisplatin. Experimental studies also confirmed these changes related with the hepatotoxicity (16,17).

In the present study, a significant increase of MDA production was observed in the liver tissue extracts in the cisplatin group when compared to the control group. However, a marked decrease of oxidative stress markers was observed in the cisplatin + APO group (Table 1). This study confirmed that cisplatin leads oxidative stress by causing a marked increase in the MDA level in the liver tissue.

MDA is a lipid peroxidation product that is released specifically due to the toxic effects of active ROS. ROS is produced as a result of oxidation of unsaturated fatty acids in cell membranes (43). The finding of increased MDA production in cisplatin-induced hepatotoxicity was in line with our study.

Another important finding was that SOD, CAT and GPx activities were decreased by cisplatin (Table 1). APO treatment increased this effect of enzymes when compared with the control group. This is because it is known that the hepatocytes have enzymatic and non-enzymatic antioxidant systems in order to maintain the integrity of the biological membranes in case of oxidative stress. The imbalance between the pro- and antioxidant system plays an important role in the development of several diseases. SOD, CAT and GPx, endogenous enzymatic antioxidants protect the organism and cells against cytotoxic free oxygen radicals. Oxygen radicals are converted to H_2O_2 by SOD. Then, CAT and GPx convert H_2O_2 into H_2O and O_2. Also, GPx acts via the reduction of the GSH. Glutation reductase then recycles the GSH. The levels of these enzymes within the host increase to protect the tissues during oxidative damage. Apocynin, a significant inhibitor of NOX, uses its pathway to suppress the oxidative stress. It is shown in this study that its effect occurs by increasing this enzyme level. However, one of the explanations of this status is that probably it could not show this effect by the NOX pathway (44). Superoxide formation does not occur only through NOX pathways. According to our knowledge, there are other pathways such as xanthine oxidase, GSH and cytochrome P450 for this effect. Other pathways may also cause an increase in CAT activity (45). Apocynin may also have influenced these other pathways. In addition, GPx production is stimulated as advanced defense mechanism when SOD and CAT are insufficient (43). Our findings confirm the hypothesis that proposes that hepatotoxicity results from ROS, which impairs the antioxidant system. Our results are in line with the studies that demonstrated that lipid peroxidation and protein oxidation increased liver enzymes and decreased antioxidant system in cisplatin-induced hepatotoxicity (16,43).

The GSH cycle is the most important intracellular antioxidant defense mechanism. It is used as a substrate for the activity of several antioxidant enzymes. In particular, GPx is a glutathione-dependent enzyme. In the presence of glutathione, H_2O_2 is detoxified by GPX via conversion to H_2O and O_2 molecules. Then, GSSG is formed by receiving hydrogen from glutathione which, in turn, is converted to GSH by glutathione reductase. Decreased GPx activity leads to the accumulation of toxins by increasing the oxidative stress (16,17). In our study, cisplatin caused a reduction in GPx and GSH levels (Tables 1 and 2). This finding is in line with the previous studies (43).

In the present study, the TOS and OSI levels were increased, whereas the TAC level was decreased in the

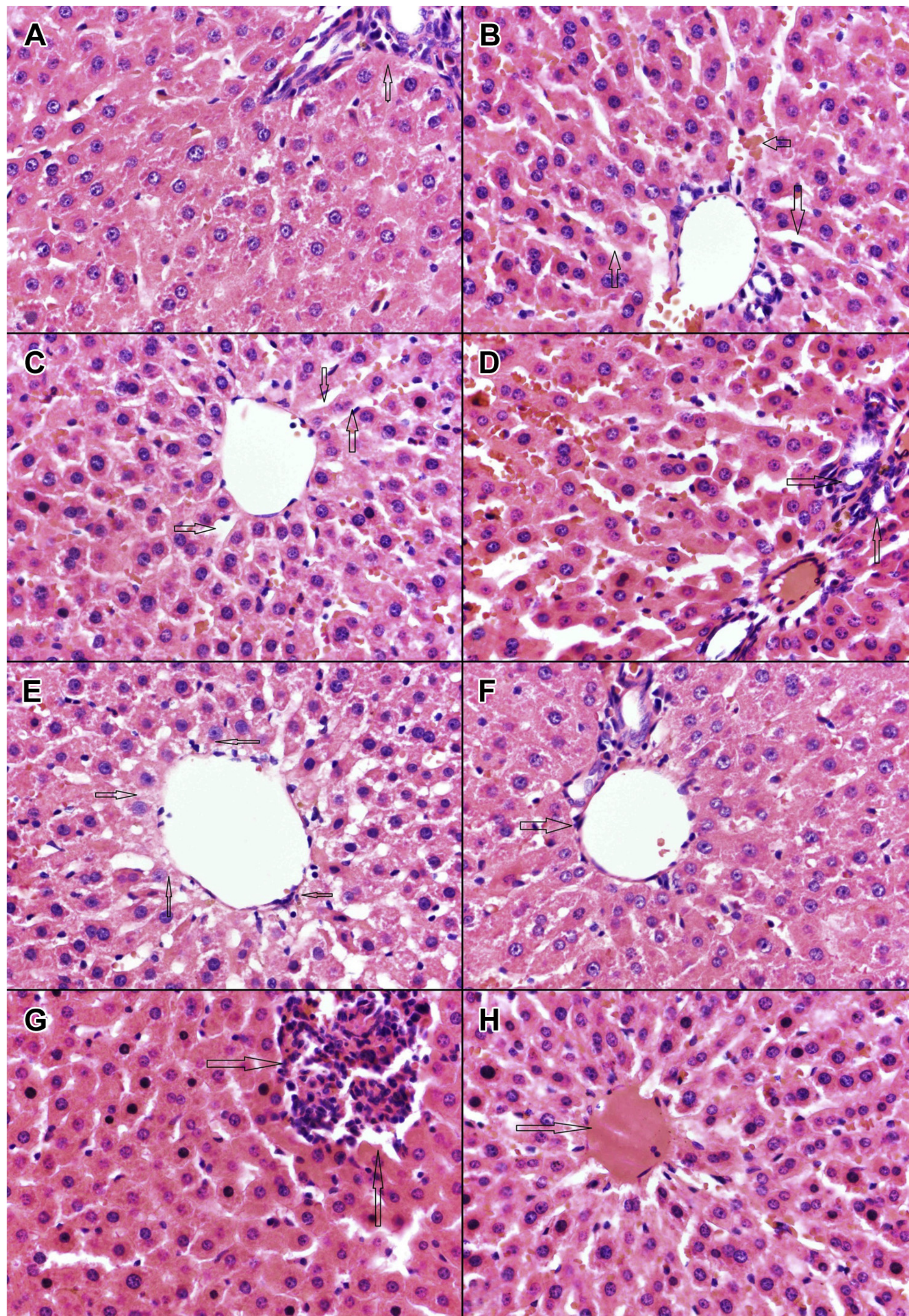

Figure 1. (A) Liver parenchyma containing central and portal veins with normal morphology in the control group. (B) Periportal sinusoidal dilatation in the cisplatin group. (C) Perivenular sinusoidal dilatation in the cisplatin group. (D) Proliferation of biliary duct in the cisplatin group. (E) Vacuolar changes in hepatocytes in the cisplatin group. (F) Liver parenchyma containing central and portal veins with normal morphology in APO-treated group. (G) Parenchymal inflammation in the cisplatin plus APO-treated group. (H) Central vein thrombosis in the cisplatin plus APO-treated group (H and E, x400). (A color figure can be found in the online version of this article.)

cisplatin group (Table 2). In the cisplatin + APO group, TOS and OSI levels were significantly decreased, whereas no significant increase was observed in the TAC level when compared to the cisplatin group. These findings suggest that cisplatin increases the oxidative stress, whereas APO decreases it (46).

Hepatotoxicity can be readily shown by liver function tests. These tests are the most sensitive biomarkers that show hepatotoxicity and cellular damage directly (47). Serum ALT level was increased in the cisplatin group, whereas it was decreased when APO was added. It is known that ALT is increased in the presence of

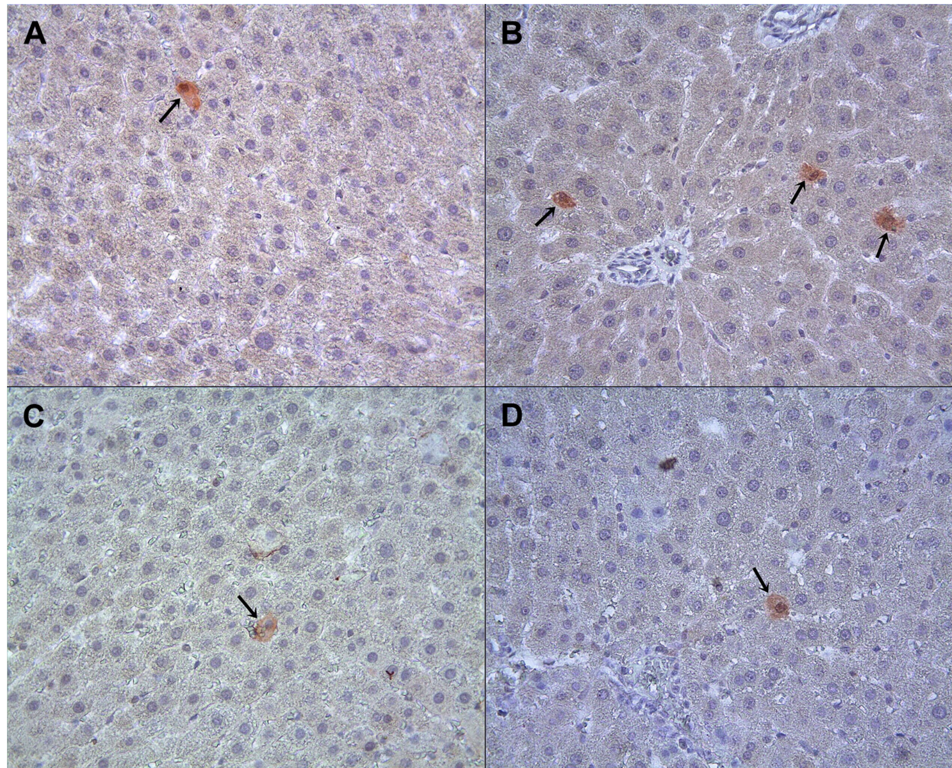

Figure 2. Arrows show caspase-3 positive cells. (A) Control group. (B) Cisplatin group. (C) APO group. (D) Cisplatin + APO group (x400). (A color figure can be found in the online version of this article.)

hepatotoxicity. This also supports that APO has antioxidant activity and decreases oxidative stress.

Morphological changes in the liver were assessed under light microscope. According to the histopathology, central vein dilatation congestion and thrombosis, perivenuler and periportal sinusoidal dilatation, parenchymal inflammation, vacuolar changes in hepatocytes and biliary duct proliferation were observed in the cisplatin group. Also, in this group, positive caspase-3 stained cells were observed immunohistochemically. These findings all support the mechanism of hepatotoxicity of cisplatin. It was observed that such changes were decreased in the group in which APO was added to the cisplatin. In a study by Li et al. it was shown that APO led a marked decrease in ROS and apoptosis by inhibiting NOX (38).

In the current study, it was shown that caspase-3 positive cells were markedly increased in the cisplatin group when compared to the control group, whereas apoptosis was decreased in the group that received APO. To further confirm the cisplatin-induced hepatotoxicity, we did not use other methods such as TUNEL assay (IHC data) and the Western blot of active-caspase-3 and PARP cleavage. Because of our limited facilities, we used only caspase-3 analysis for the execution of caspase cleavage for detecting apoptosis in light of literature reports. Parlakpinar et al. reported that it has become increasingly clear that TUNEL assay is prone to false-positive and -negative results (48). TUNEL staining detects single-strand DNA breaks as well as double-strand DNA breaks. Therefore, TUNEL positivity is seen in both necrotic and apoptotic myocytes. Detection of active components of apoptotic pathways such as caspases can help to confirm the findings (48,49). Based on this relationship, Parlakpinar et al. reported that the activation of caspase-3 which, in turn, cleaves multiple cellular proteins, resulted in cell death (48). Thus, caspases are universal effectors of apoptosis and can be considered as the central executioners of the apoptotic machinery. Inhibition of their activities delays the retards or even prevents apoptosis. Our finding is in accordance with the above mentioned report and also with a study by Choi et al. who also reported the anti-apoptotic effect of APO. Also, the histopathological changes were found to be in accordance with increased MDA, TOS, OSI and serum ALT levels in combination with decreased SOD, CAT and GPx contents in the cisplatin group and improvements in these parameters in the cisplatin + APO group (50).

However, cisplatin has important adverse effects such as nephrotoxicity, neurotoxicity and ototoxicity. In previous studies, it was shown that cisplatin-induced ototoxicity and nephrotoxicity are closely related with the increment of ROS. It was shown that the ROS was generated, in part, through the activation of the NADPH oxidase, which plays an essential role in cisplatin toxicity (51). As is well-known, cisplatin also causes depletion of enzymatic and

non-enzymatic antioxidant system (GSH; SOD, CAT, GPx and glutathione reductase) and increment of oxidant system (MDA, toxic lipid peroxides). Many agents have been studied to evaluate their protective effects against cisplatin-induced ototoxicity and nephrotoxicity such as apocynin. The beneficial and protective effect has been shown in previous experimental studies (50,52). In these related studies it was shown that apocynin has antioxidative effects and prevents apoptotic features.

We compared the data obtained with those of the literature showing that other pure antioxidant compounds (chrysin, thymoquinone, crocin, sulforaphane, caffeic acid phenethyl ester and ellagic acid) are able to attenuate cisplatin-induced liver damage and oxidative stress. It is possible that the beneficial effects of these antioxidants were nearly similar. All of these pure antioxidant compounds have an effect on liver by strengthening the antioxidant defense system by reducing reactive oxygen species and increasing antioxidant enzyme activities (7,36,40,47,53,54).

In each of these findings beneficial actions of APO were attributed to its functions as an antioxidant. In addition to being a free radical scavenger, APO may also reduce the generation of reactive free radicals by inhibiting NADPH-oxidase (pro-oxidant trigger pathways). Due to our limited facilities in the current study, we attempted to assay the major oxidative and antioxidant parameters. Therefore, other oxidative stress parameters like conjugated dienes, lipid hydroperoxides and protein carbonylation, which reflect the lipid and protein oxidation and total sulfhydryl content in the liver, could not be evaluated.

In conclusion, although the mechanisms underlying the cisplatin-induced hepatotoxicity are complex, free radicals and oxidative stress play important roles. It is essential to control the formation and activity of the reactive oxygen species because they impair cellular functions. The present study showed that APO can decrease cisplatin-induced hepatotoxicity via its antioxidant and free radical scavenger effects at both histopathological and biochemical levels. We think that APO can be used as a protective agent against cisplatin-induced hepatic injury. However, further clinical studies are needed to demonstrate the protective effects of APO against cisplatin-induced hepatotoxicity.

Conflict of Interest

All authors declare no conflict of interest.

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