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POMEGRANATE SEED EXTRACT ATTENUATES CHEMOTHERAPY-INDUCED LIVER DAMAGE IN AN EXPERIMENTAL MODEL OF RABBITS

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Abstract

The aim of this study was to determine whether antioxidant pomegranate seed extract (PSE) has a preventive effect on cisplatin-induced hepatotoxicity. Rabbits were divided into 3 groups (n=6):1-Control group (0.9 % saline. i.p) 2-Cisplatin group (a single dose of cisplatin (5 mg/kg, i.p) 3- A single dose of cisplatin (5 mg/kg, i.p) + PSE (250 mg/kg/day, i.p) for 6 consecutive days before and 6 consecutive days after a single intraperitoneal dose of 5 mg/kg body weight cisplatin. Liver function enzymes and malondialdehyde (MDA) levels were found significantly higher in cisplatin group compared to control. Liver catalase (CAT) and glutathione peroxidase (GSH-P_X) activities decreased with cisplatin treatment but glutathione (GSH) level was increased. In cisplatin + PSE group, liver function enzyme activities and tissue MDA levels were found lower than cisplatin group. PSE ameliorated cisplatin-induced pathological changes. As a result it was demonstrated that PSE has protective effects against cisplatin hepatotoxicity in rabbit.

Key words: Pomegranate seed extract, Antioxidant effect, Cisplatin, Hepatotoxicity, Rabbit.

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INTRODUCTION

Cisplatin (*cis*-diamminedichloroplatinum II, CDDP) is a potent drug which is widely used in the treatment of variety of solid tumors (8). However, its clinical usage in high doses is restricted in practice because of its strong side effects in the liver, the kidneys, and other organs (25, 39). CDDP causes the generation of reactive oxygen species (ROS), depletion of GSH levels, and inhibition of antioxidant enzyme activity in these tissues. Additionally, many studies show that CDDP induces oxidative stress, lipid peroxidation, and DNA damage (29, 21).

The liver is the main organ responsible for multitude of essential functions and plays an essential role in metabolism of foreign compounds entering the body (3). Although cisplatin-induced nephrotoxicity has been very well documented in clinical oncology, hepatotoxicity has been rarely characterized, and is less studied. It is known that cisplatin is significantly taken up in human liver and those high doses of the drug produces hepatotoxicity (16, 43). Hepatotoxicity is a less- known aspect of cisplatin treatment, and there is little information about the underlying mechanism (13). Generally liver toxicity of cisplatin is characterized by mild to moderate elevation of serum transaminases and less frequently, by a mild elevation of alkaline phosphatase (10). The enzymes L- alanine aminotransferase (L-ALT), L-aspartate amino transferase (L-AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH), are often used in assessing the integrity of the liver (27).

Plants, vegetables, herbs and spices used in folk and traditional medicine have been accepted currently as one of the main sources of chemopreventive drug discovery and development (2). Pomegranate (*Punica granatum* L., Punicaceae) is one of the oldest known drug. It is mentioned in the Ebers papyrus of Egypt written in about 1550 BC (33). The Pomegranate Fruit Extract, because of its robust content of polyphenolic flavonoid antioxidants, is expected to enhance the biological actions of naturally produced NO in vivo. Regular pomegranate juice administered to hypertensive patients caused a significant drop in blood pressure (4), a reduction in carotid plaque development (5) and an improvement of stress-induced myocardial ischemia in patients who have coronary heart disease (36). From a pathogenic point of view, we have shown that regular pomegranate juice reduced the expression of oxidation-sensitive genes at the sites of perturbed shear stress and protected against high-prone atherosclerotic areas in hypercholesterolemic mice (14).

Antioxidants, in general, are compounds which dispose, scavenge, and suppress the formation of ROS and lipid peroxidation. Among the well known biological antioxidants, glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT), superoxide-dismutase (SOD) have a significant role as a suppressor or scavenger of free radicals (38, 44). It has been reported that oxidative stress through the generation of reactive oxygen species (ROS), decreased antioxidant defense system including antioxidant enzymes (35) and non-enzymatic molecule reduced glutathione (GSH) are major alterations in cisplatin toxicity (47). Many investigators have reported that pomegranate and its derivatives have free radical scavenger and potent antioxidant activity (6, 32). Pomegranate juice has become more popular because of the attribution of important biological actions (22). Pomegranate juice, peel, seeds-all have a potent antioxidant activity. Kaur et al. (2006) suggested that pomegranate flowers too boast an enormous antioxidant activity (18). Antioxidant potential of pomegranate juice and extracts are attributed to their high polyphenolics content including ellagic acid and ellagitannins (19).

The aim of this study was to investigate possible protective effects of PSE supplementation on CDDP-induced

oxidative organ injuries, and its effects on the levels of antioxidant enzymes and lipid peroxidation, as well as histological changes.

MATERIALS AND METHODS

Animals and experimental design

18 healthy male New Zealand white rabbits, weighing 2.5~3 kg, were used throughout this study. The animals were obtained from the Veterinary Control and Research Institute, Elazig, Turkey. The animals were kept under standard laboratory conditions (12-h light:12-h dark and 24±3 ◦C). The protocol of this study was approved by the Veterinary Control and Research Institute Ethics Committee. The rabbits were fed with standard commercial rabbit chow (pellet form, in the sack, Elazig Food Company). Feed and water were provided ad libitum.

The rabbits were divided into three groups; each group containing six rabbits. The first group of rabbits served as control and was administered a single intraperitoneal dose of 0.9% saline. The second group of rabbits was treated with cisplatin. Cisplatin was intraperitoneally (i.p) injected to animals at a single dose of 5 mg/kg body weight. The dose of CDDP (5 mg/kg b.w.) was selected on the basis of its effectiveness in inducing hepatotoxicity (45, 30). The third group (n = 6) of rabbits was treated with pomegranate seed extract (pomegranate seed extract were dissolved in water and administered to animals by gavage at the dose of 250 mg/kg body weight) for 6 consecutive days before and 6 consecutive days after a single intraperitoneal dose of 5 mg/kg body weight cisplatin injection (7).

Sample collection and biochemical assays

At the end of the experiments, animals of each group were decapitated under slight ether anaesthesia and samples of liver tissues from each group were collected for biochemical and histopathological examination. Blood samples were collected into tubes and centrifuged at 3000×g for 10 min. Serum was separated and then stored at−20°C until analysed.

The liver tissues were homogenized in glass-glass homogenizer with a buffer containing 1.5 % potassium chloride to obtain 1:10 (w/v) whole homogenate. Concentrations of malondialdehyde, as proceeding lipid peroxidation, were measured in the homogenate. Homogenates were centrifuged at 5000 rpm, 20 min, at + 4 ºC to determine of glutathione level, catalase and glutathione peroxidase activity and the supernatant was subjected to enzyme assays immediately.

Determination of tissue MDA and GSH levels

Lipid peroxidation (as malondialdehyde) levels in liver homogenate were measured with the thiobarbituric-acid reaction by the method of Placer et al.1966 (31). The values of malondialdehyde were expressed as nmol/g tissue. The glutathione contents in liver were measured at 412 nm using the method of Sedlak and Lindsay 1968 (37). The levels of glutathione were expressed as nmol/g protein for liver tissue.

Determination of tissue CAT and GSH-P_x activity

The glutathione peroxidase activity was determined according to the method of Lawrence and Burk 1976 (23). The protein concentration was also measured by the method of Lowry et al. 1951 (24).

The glutathione peroxidase activity was expressed as IU/g protein for liver tissue. The liver tissue catalase activity was determined by measuring the decomposition of hydrogen peroxide at 240 nm, according to the method of Aebi 1983(1) and was expressed as k/g protein, where k is the firstorder rate constant.

Determination of liver functions

Serum AST, ALT, ALP and GGT levels were measured to evaluate the liver function. All biochemical assays were performed by using an autoanalyzer (Olympus AU 600, Japan).

Histopathologic Evaluation

At the end of the experiment, necropsy of the rabbits was done, after they were decapitated under slight ether anaesthesia, and their liver tissue samples were fixed in 10 % buffered neutral formalin. Parafin-embedded blocks were prepared after the tissues were subjected to routine processes. The cross section (thickness 5 µ) from the blocks were stained with the Hematoxylin-Eosin (H-E) method and examined under an light microscope (Olympus BX51). 10 microscopic fields were examined regarding periportal cell infiltration, necrosis, sinusoidal congestion, acute cell swelling, hepatic cord disorganisation, hepatic steatosis and capsule fibrosis. Every fields were evaluated as severe (+++), moderate (++), mild (+) and none (-).

Statistical analysis

Data are presented as mean ± Standard error of means (SEM). One-way analysis of variance and post hoc Duncan's test were used to determine the differences between the groups in terms of all studied parameters using the SPSS/PC computer program (version 12.0; SPSS, Chicago, IL, USA). Data for histopathological scores were abnormally distributed ($P<0.05$). Therefore, Kruskal–Wallis analysis of variance and Mann–Whitney U test were performed for histopathological scores. Differences were considered significant if the P value was less than 0.05.

RESULTS

The changes in MDA levels and GSH, GSH-Px, and CAT activity in the liver are shown in Table 1. When compared to the control groups, the MDA levels in the liver were significantly ($p < 0.001$) higher in groups administered with CDDP. On the other hand, this increase was attenuated by pre-treatment with PSE. The CDDP-treated rabbits showed significantly reduced GSHPx ($p < 0.05$), CAT activity ($p < 0.01$) but increased GSH levels ($p < 0.05$), in the liver tissue when compared with the control groups. Pre-treatment of rabbits with PSE alleviated the CDDP-induced decreases in GSH-Px, and CAT activity in the liver tissue (Table 1).

Cisplatin also caused significant increases in serum ALT, AST, ALP and GGT levels when compared to control levels (Table 2). Thus, these data indicate that a single intravenous injection of single dose of 5 mg/kg body weight cisplatin impairs liver functions. PSE was also found to be effective to reverse cisplatin-induced changes in serum ALT, AST, ALP and GGT levels (Table 2).

The histopathologic changes observed in the liver are summarized in Table 3. The livers in the control group had

Table 1. Effects of cisplatin (5 mg/kg, intraperitoneally) and Pomegranate seed extract (PSE, *per os*, 250 mg/kg for 6 days before and 6 days after cisplatin injection) treatments on serum activities of liver enzymes in rabbits.

	MDA nmol/g tissue	CAT k/g protein	GSH nmol/g tissue	GSH-P_X IU/g tissue
Control	6.83±0.39^{a}	303.60±6.64^{b}	1.86±0.13^{a}	30.52±2.89^{b}
Cisplatin	10.61±0.44^{b}	232.60±16.80^{a}	2.32±0.16^{ab}	19.83±0.70^{a}
Cisplatin+PSE	6.16±0.72^{a}	347.80±26.88^{b}	2.74±0.31^{b}	25.50±2.22^{ab}

PSE: Pomegranate seed extract; MDA: malondialdehyde; GSH: reduced glutathione; GPx: glutahione peroxidase; CAT: catalase. Different superscripts a,b,c in the same column indicate significant difference ($p < 0.05$ or more) between groups. Results are expressed as mean ± standard error of the mean (SEM).

Table 2. Effects of cisplatin (5 mg/kg, intraperitoneally) and Pomegranate seed extract (PSE, *per os*, 250 mg/kg for 6 days before and 6 days after cisplatin injection) treatments on antioxidant / oxidant equilibrium in liver homogenates in rabbits.

	AST (U/L)	*ALT(U/L)*	*ALP(U/L)*	*GGT(U/L)*
Control	22.60±2.03^{a}	18.40±1.16^{a}	66.20±2.95^{a}	2.80±0.37^{a}
Cisplatin	41.60±2.13^{b}	39.20±1.20^{b}	116.80±11.11^{b}	4.60±0.24^{b}
Cisplatin+PSE	20.80±1.06^{a}	20.80±0.66^{a}	45.60±2.08^{a}	2.00±0.31^{a}

PSE: Pomegranate seed extract; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatases; GGT: γ-glutamtyl transferase. Different superscripts a,b in the same column indicate significant difference ($p < 0.001$) between groups. Results are expressed as mean ± standard error of the mean (SEM).

their normal histological appearances (Picture C). Some histological changes were identified in the groups which were treated with both Cisplatin and PSE.

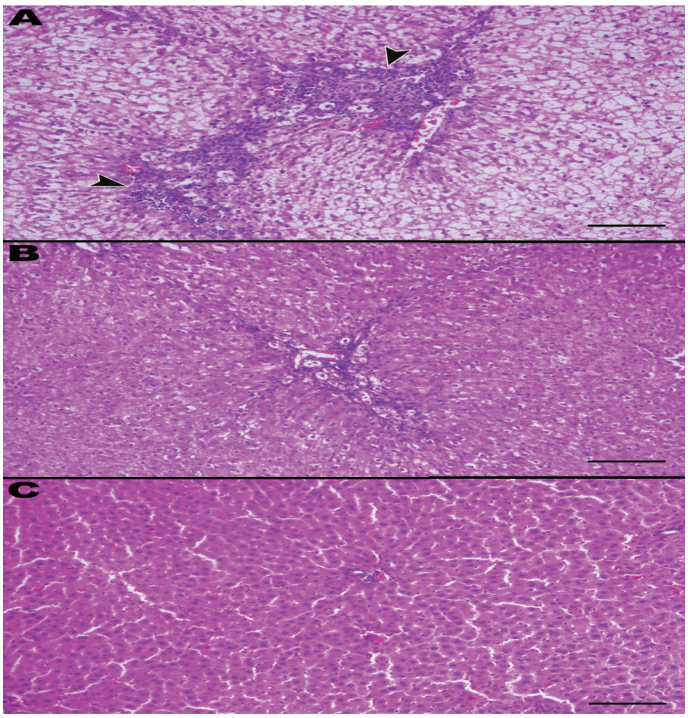

Figure 1. Picture A- Severe acute cell swelling with mononuclear cell infiltration Cisplatin group. X 20. Picture B- Moderate degenerative changes in hepatocytes. Cisplatin+ PSE group. X 20. Picture C- Normal histological appearance of liver, Control group. X 20.

The most noteworthy change in the appearance of liver in the group that was treated with Cisplatin only was the numerous mononuclear cell infiltrations along with Kupffer cell activations in periportal areas, acute cell swelling and cardiomegaly in hepatocytes (Picture A). In addition, capsular fibrosis, necrosis characterized by the changes in the cell and the hepatocytes with two cells were the other significant changes.

It was determined that the numbers of periportal cell infiltrations and hepatocytes with two cells in the group which were treated with both Cisplatin and PSE together were lower than they were in the group which were treated with only Cisplatin (Picture B) In this group, the severity of necrotic changes were similar in the group received cisplatin alone. In both groups, there was disorganization in hepatic cord (Figure 1).

DISCUSSION

Cisplatin is one of the most active cytotoxic agents in the treatment of cancer. Liver and kidney toxicity are major complications, which are dose-limiting factors for cisplatin therapy (41).

The results of our study suggest that cisplatin could induce oxidative stress and decrease antioxidant defense mechanism leading to lipid peroxidation in the liver. A single dose of cisplatin (5 mg/kg) in the current study resulted in hepatotoxicity as evidenced by biochemistry and histopathology (13). In the present study, the level of liver MDA in the CDDP treated group was significantly higher compared with their levels in the controls. Increased MDA levels indicated that lipid peroxidation, mediated by ROS, was an important contributing factor in the development of CDDP-mediated tissue damage (11,12). However, pretreatment with PSE significantly prevented CDDP-induced lipid peroxidation in the liver.

Table 3. Effects of cisplatin (5 mg/kg, intraperitoneally) and Pomegranate seed extract (PSE, *per os*, 250 mg/kg for 6 days before and 6 days after cisplatin injection) treatments on liver histology in rabbits.

LESIONS	GROUPS				
	Cisplatin	**Cisplatin +PSE**	**Control**	**SE**	**P**
Periportal cell infiltration	2.00 ± 0.26 [a]	1.33 ± 0.33 [a]	0.00 ± 0.00 [b]	0.24	0.001
Necrosis	2.17 ± 0.31 [a]	2.00 ± 0.26 [a]	0.00 ± 0.00 [b]	0.27	0.001
Sinusoidal congestion	1.17 ± 0.17 [a]	0.83± 0.17 [a]	0.00 ± 0.00 [b]	0.14	0.001
Acut cell swelling	2.00 ± 0.45 [a]	1.50 ± 0.57 [a]	0.00 ± 0.00 [b]	0.31	0.01
Hepatic cord disorganization	2.33 ± 0.21 [a]	2.00 ± 0.26 [a]	0.00 ± 0.00 [b]	0.27	0.001
Fatty degeneration	0.83 ± 0.31 [a]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	0.14	0.001
Capsular fibrogenesis	1.00 ± 0.26 [a]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	0.14	0.001

PSE:Pomegranate seed extract. Different superscripts a,b in the same row indicate significat difference ($p < 0.05$ or more) between groups. Results are expressed as mean ± standard error of the mean (SEM).

GSH is one of the most important molecules, for maintaining cell integrity and participation in cell metabolism (26). The role of GSH, which are non-protein thiols in the cells, in the formation of conjugates with electrophilic drug metabolites (most often formed by cytochrome P450-linked monooxygenase) is well established (9). GSH is an important part of the non-enzymatic antioxidant system, and it was known to play important role in the elimination of cisplatin (15). In this study, GSH levels in the liver tissue of rabbits treated with CDDP were higher than the control group's. This status may be explained by the fact that GSH synthesis has been shown to be induced in cells exposed to oxidative stress as an adaptive process. In the same way, Tian et al. (1997) and Yilmaz et al. (2006) suggested that under oxidative stress conditions, there may be positive regulation in the glutathione biosynthesis, resulting in the increased level of GSH contents (40,46).

The reduced liver GSH-Px and CAT activity in animals treated with CDDP alone were compared with the control group's levels of activity. These observations indicated that the mechanism of liver toxicity induced by CDDP in animals is partially related to the depletion of liver antioxidant systems. Treatment with PSE before the CDDP challenge could significantly prevent the depletion of liver antioxidant systems. Furthermore, in this study, it was observed that levels of GSH-Px and CAT in the PSE+CDDP treated groups were higher than in the CDDP group. These results suggested that PSE has a supporting effect on the antioxidant system because of increases in GSH-Px and CAT levels. Recently, it has been demonstrated that PSE prevents CDDP-induced hepatotoxicity (11). Similar results have been reported by Kart et al. 2010, Koc et al. 2005 (20, 21). Mansour et al. 2006 suggested that CDDP caused low GSH-Px and CAT levels in liver tissue (28). These reports and results of our study suggest that cisplatin could induce oxidative stress and decrease antioxidant defense mechanism leading to lipid peroxidation in the liver.

In the present study, cisplatin administration caused severe damage in the liver, as assessed microscopically. The liver morphology was characterized by severe activation of Kupffer cells, degenerated hepatocytes and sinusoidal congestion. The histological results are presented in Table3.

The efficacy of any hepatoprotective herb is essentially dependent on its capacity of either reducing the harmful effects or maintaining the normal physiologic function which has been disturbed by hepatotoxic agents. Exogenous and endogenous protective agents with antioxidant properties were reported to show some protective effects in cisplatin-induced hepatotoxicity. PSE is one promising agent against various toxicities associated with oxidative stress and peroxidative damage (34). In our current study, PSE treatment against cisplatin toxicity significantly reduced the elevated MDA level and also normalized tissue GSH-P_X and CAT activity, but cisplatin-induced increase in GSH level was reduced by PSE treatment.

Measurement of the activities of serum marker enzymes, like AST, ALT and ALP, can make assessment of liver function (42). In this study the hepatoprotective effect of PSE was evaluated by measuring levels of serum marker enzymes such as (alkaline phosphatise) ALP, (alanine aminotransferase) ALT, (aspartate aminotransferase) AST and (gamma-glutamyltransferase) GGT levels in rabbit serum. Deteriorations of liver function tests (serum ALP, ALT, AST, GGT) revealed hepatic dysfunction in cisplatin group. Similar results have been reported by Kart et al. 2010 (20), for liver tissue in which CDDP caused high ALT, AST levels. Decreased serum AST, ALT, ALP and GGT levels in response to PSE treatment indicate reduced hepatocellular damage.

The results of the present study demonstrate that PSE treatment of rabbits markedly improves cisplatin-induced liver dysfunction and organ damage as confirmed by microscopic examination and biochemical assays.

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