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Full Length Research Paper

Effects of pomegranate seed extract on liver paraoxonase and bcl-x_L activities in rats treated with cisplatin

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The aim of the present study was to investigate the protective effect of pomegranate seed extract (PSE) on the changes caused by cisplatin (CP) in rat liver paraoxonase and bcl-x_L activities. Twenty-four Sprague-Dawley rats were randomly divided into four groups of six animals: (1) Control; (2) PSE: Treated for 15 consecutive days by gavage with PSE (300 mg/kg/day); (3) CP: Injected intraperitoneally with cisplatin (7 mg/kg body weight, single dose); and (4) PSE+CP: Treated by gavage with PSE 15 days after a single injection of CP. Blood and liver tissue samples were taken from each animal after experimental procedures. PON-1 paraoxonase and arylesterase activities and malondialdehyde (MDA) levels were estimated from liver homogenates; the liver tissue was also immunohistochemically examined for anti-apoptotic activity (bcl-x_L). The reduction caused by cisplatin in paraoxonase and arylesterase activities could be prevented at a significant level in the rats given a pre-treatment with PSE before the injection of cisplatin. Also, PSE significantly attenuated the cisplatin-induced structural alterations in the liver tissue, and increased anti-apoptotic hepatocyte numbers in the interlobular area and around the central vein of liver, but they were not observed in CP treated rats. PSE may be used as a preventive agent against the cisplatin-induced hepatotoxicity in patients receiving chemotherapy medications.

Key words: Arylesterase, cisplatin, paraoxonase, pomegranate, anti-apoptosis.

INTRODUCTION

Pomegranate *(Punica granatum*) fruit has been consumed extensively in the form of fresh fruit, concentrate juice and pomegranate sour in salads in Turkey and the Mediterranean region) (Tezcan et al., 2009). The pomegranates are found to be a rich source of polyphenolic compounds that include flavonoids (anthocyanins, catechins and other complex flavonoids) and hydrolyzable tannins (punicalin, pedunculagin, punicalagin, gallagic acid and ellagic acid esters of glucose) which account for 92% of their antioxidant activities (Zahin et al., 2010). In recent years, many research results have been published about the beneficial effects of pomegranate fruit. For example, it has been reported that consumption of pomegranate juice may be helpful against coronary heart disease (Sumner et al., 2005) and Alzheimer's disease (Singh et al., 2008). Also, pomegranate extract significantly improve arteriogenic erectile dysfunction (Zhang et al., 2011) and sperm quality in male patients (Turk et al., 2008b). In addition, the more remarkable scientific articles are available on cancer chemoprevention by pomeganate extracts and active compounds *in vitro*, as well as in experimental animal models (Adhami et al.,

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2009). Cisplatin (cis-diamine-dichloroplatinum) is one of the principal drugs used to treat various types of cancers, including lung cancer, ovarian cancer and testicular cancer (Gottfried et al., 2008; Turk et al., 2008a). However, the use of high-dose cisplatin can lead to serious side effects. The toxic effects which occur primarily in the liver and other organs restrict the clinical use of cisplatin (Jiang and Dong, 2008; Liao et al., 2008). Cisplatin toxicity may occur with different mechanisms. Studies have shown that cisplatin leads to the formation of reactive oxygen species, lipid peroxidation and DNA damage (Cayir et al., 2009). Therefore, an important point that should be considered during treatment with cisplatin is that consumption of dietary antioxidants may be useful against free radical damage.

Serum paraoxonase-1 (PON-1) is a calcium-dependent ester hydrolyse which has got both paraoxonase and arylesterase activities. It is one of the three members of the paraoxonase family (PON-1, PON-2, PON-3). Serum PON-1 is synthesized mainly in the liver and secreted into the blood circulation (Draganov et al., 2005; Rosenblat et al., 2006). PON-1 has anti-oxidative properties, which are associated with the enzyme's capability to protect LDL and HDL from oxidation, to decrease the lipid peroxidation caused by the free radicals on the cell membranes and lipoproteins and to slow the development of atherosclerosis (Costa et al., 2005; Ferretti et al., 2003; Toker et al., 2009; Yildirim et al., 2007). Aviram et al. (2000) reported that the consumption of pomegranate juice by healthy human subjects for 2 weeks significantly reduced the oxidation of both LDL and HDL and increased the activity of serum paraoxonase. In addition, it has been shown in a recent study that PON-1 expression in hepatocytes is upregulated by pomegranate polyphenols (Khateeb et al., 2010).

The consumption of dietary antioxidants such as pomegranate juice, blueberry juice and green tea inhibit oxidative stress and prevent free radical injury (Zhang et al., 2011). Thus, we envisage that pomegranate seed may indicate protective antioxidant activity which might have promising therapeutic potential against the oxidative organ damage caused by cisplatin. For this purpose, the antioxidant effects of pomegranate seed extract have been analysed with the PON-1 paraoxonase and arylesterase enzyme activities measured in the liver tissue samples. Further, the hepatotoxicity associated with cisplatin treatment and the protective effects of pomegranate extract were examined histologically.

MATERIALS AND METHODS

Animals and experimental procedure

After obtaining approval from the local ethics committee of animal experiments in Ataturk University, we used 24 female adult Sprague-Dawley rats weighing approximately 200 g. Before starting the experimental protocols, the rats were randomly divided into four groups of six animals: Control group (Group C), the group that took pomegranate seed extract (Group PSE), the group that took cisplatin (Group CP) and the group that took pomegranate seed extract+cisplatin (Group PSE+CP). The animals were kept in the metal cages with a temperature of 22 to 24°C and a 12-h light / dark cycle during the study. The rats in the control group were fed with standard rat food and tap water, while the PSE group rats were given 300 mg/kg/day pomegranate seed extract (Balen Pomegranate Seed Extract Capsule, Arı Mühendislik Company, Ankara, Turkey) through the oragrastric tube for 15 days. Group CP rats were given a single dose of cisplatin (7 mg kg^{-1} body weight i.p.). Group PSE+CP rats were given PSE 300 mg/kg/day through oragrastic way for 15 days and then a single dose of cisplatin (7 mg kg^{-1}, i.p.). The doses of cisplatin and PSE used in this study were selected in accordance with the previous studies (Al-Majed, 2007; Cayir et al., 2011).

Taking of the tissue and blood samples and biochemical measurements

Twenty-four hours after the end of the study, the animals in the control and experimental groups were anaesthetized with an i.p. injection of 60 mg sodium pentobarbitone per kg of body weight. Then the rats were killed by taking blood samples from the heart, and liver tissues were immediately removed. Blood samples were centrifuged for 5 min. at 3500 g and their serum parts were separated and stored at -80°C until they were analyzed. Tissue samples were cleaned by the solution of isotonic NaCl at ice cold for the removal of bloody spots, and then they were dried with blotting paper.

About 300 mg liver tissue was weighed for each rat and homogenized within the tampon solution at ice cold buffer (50 mM Tris-HCl, pH 8.0, containing 2 mM $CaCl_2$) (OMNI TH homogenizer, Warrenton, VA, USA). Tissue homogenates were centrifuged at 15,000 g for 15 min. (4°C) and the supernatant part was kept for biochemical analysis at -80°C. Tissue protein levels were determined with Bradford method (Bradford, 1976) and the serum ALT level was determined with Roche Cobas biochemical autoanalyzer by using a commercial kit (Roche Diagnostics, Mannheim, Germany).

Measurement of PON-1 paraoxonase and arylesterase (ARE) activities

PON-1 and ARE activities were assessed by methods described previously (Beltowski et al., 2005; Eckerson et al., 1983), with some modifications. The original method was made semi-automatized with the adaptation of 96-well microplate and thus in a short time the analysis of a multitude of samples was made possible. The kinetic measurements were conducted with the use of spectrophotometric microplate reader (PowerWave XS, Bio-Tek Instruments, Inc.) and its software program (KC Junior software, Bio-Tek Inc.).

PON-1 measurement was realized at 405 nm and ARE at 270 nm. As ARE measurements were made at ultraviolet wavelength (270 nm), the 96-well ultraviolet plate was used for assays. For the measurements of PON-1 and ARE activities, diethyl-p-nitrophenyl phosphate (Sigma Co, UK) and phenyl acetate (Sigma Co, UK) were used as the substrates, respectively. Molar absorption coefficients were used in the calculation of PON-1 and ARE activities (17100 and 1310 M^{-1} cm^{-1}, respectively). One unit for PON-1 activity was defined as 1 nmol 4-nitrofenol/mL serum/min and that for ARE activity was defined as 1 mmol fenol/mL serum/min. Tissue-specific activities of PON-1 and ARE in liver were calculated and results are expressed as U/mg protein.

Table 1. Biochemical parameters in the study groups (values are mean ± SD).

Parameter	Groups			
	C (n=6)	PSE (n=6)	CP (n=6)	PSE+CP (n=6)
Serum ALT activity (U/L)	32.8 ± 4.6	33.2 ± 4.1	50.5 ± 5.7[b]	45.9 ± 8.7[a]
Liver MDA (nmol/g protein)	36.9 ± 8.1	34.1 ± 9.7	59.8 ± 16.3[c]	50.2 ± 13.7[d]
Paraoxonase (U/mg protein)	8.1 ± 1.6	10.2 ± 1.8	5.2 ± 1.1[a]	6.8 ± 1.3[e]
Arylesterase (U/mg protein)	6.6 ± 0.5	8.7 ± 1.2 [a]	4.2 ± 0.7[b]	5.6 ± 1.1[f]

[a], p<0.005, [b], p<0.001, [c], p<0.05, [d], p=0.069, [e], p=0.178 and [f], p=0.073 versus control group (C).

Measurement of malondialdehyde (MDA) levels

The levels of MDA in liver tissue were used as the indicator of oxidative stress (lipid peroxidation), and were determined spectrophotometrically according to the method described previously (Ohkawa et al., 1979). A mixture of 20% acetic acid, 8.1% sodium dodecyl sulphate and 0.9% thiobarbituric acid was added to 0.2 ml of each liver homogenate, and then distilled water was added to the mixture to bring the total volume up to 4 ml. This mixture was incubated at 95°C for 1 h. After incubation, the tubes were cooled by tap water and 1 ml distilled water plus 5 ml n-butanol/pyridine (15:1, v/v) were added, followed by mixing. The samples were centrifuged at 4000 × g for 10 min. The supernatants were removed, and absorbances were measured with respect to a blank at 532 nm. Total thiobarbutiric acid reactive substances were expressed as MDA. 1,1,3,3-Tetraethoxypropane (Aldrich T9889) was used as the standard and MDA level was expressed as nmol/g protein.

Histochemical and immunohistochemical examination

The livers tissue was fixed in 10% buffered neutral formalin, and embedded in paraffin. The paraffin blocks were cut 5 to 7 µm thick and stained with Mallory's triple stain modified by Crossman. Stained specimens were examined under a light microscope. Anti-apoptotic cells were determined with immunohistochemical method (streptavidin-biotin-peroxidase staining). For immunohistochemistry examination, primary antibody bcl-x_L (dilution: 1/50 Santa cruz-sc-8392), and biotinylated secondary antibody (DAKO-Universal LSAB Kit-K0690) were used. The binding sites of antibody were visualized with DAB (Sigma), and evaluated by high-power light microscopic examination (Nikon i50). All anti-apoptotic staining cells were estimated with an image processing system (Kameram SLR, 1.6.1.0, Mikro Sistem Ltd. Şti., Turkey). For each specimen, bcl-x_L immunreactivity was examined in 10 randomly selected areas of approximately X20 objective.

The microscopic scoring of sections was carried out by a histopathology laboratory technician and histologist. This scale has composed to A = weak in ≤ 25% of tissue; B = mild in ≤ 50% of tissue; C = moderate in ≤ 75% of tissue; and D = very strong in ≥ 75% of tissue. The average degeneration intensity was calculated as [(A × 1) + (B × 2) + (C × 3) + (D × 4)]/(A + B + C + D) and reported as follows: + = 0.00-1.00; ++ = 1.01-2.00; +++ = 2.01-3.00; and ++++ = 3.01-4.00. The scores were derived semi-quantitatively using light microscopy on the preparations from each animal, and were reported as follows: none = -, mild = +, moderate = ++, severe = +++, and very strong = ++++.

Statistical analysis

Statistical analyses were performed with PASW Statistics 18 (SPSS, Inc.). Kruskal–Wallis and Mann–Whitney U non-parametric tests were used to compare the biochemical parameters among study groups. A p value less than 0.05 was considered statistically significant. Data were expressed as mean ± S.D.

RESULTS

Biochemical results

Serum ALT level was used as an indicator of the liver damage caused by cisplatin. The results showed that cisplatin caused a significant increase in serum ALT levels when compared with the control groups (p<0.001). Pomegranate seed consumption partially prevented the increase in the serum levels of ALT caused by cisplatin administration, but this decrease did not differ statistically compared with group CP (p=0.317). In addition, in the cisplatin group, the levels of liver MDA were evidently higher than in the control and other groups.

However, the liver MDA levels of the PSE+CP group showed less increase than those in the rats who received only cisplatin, and this decrease in MDA level of the PSE+CP group did not differ statistically compared with controls (p=0.069), suggesting that pomegranate seed extract possesses antioxidant effect against lipid peroxidation.

These results were presented in Table 1. PON-1 paraoxonase and arylesterase activities were measured in liver homogenate. Treatment of normal rats with pomegranate seed extract (300 mg/kg/day, Group PSE) for 15 days resulted in a marked increase in the PON-1 activities in the liver tissue of rats. The most striking increase was in the PON-1 arylesterase activity. These increases in PON-1 paraoxonase and PON-1 aryesterase activities were about 26% (p=0.058) and 32% (p<0.001), respectively, compared to untreated rats (Group C). On the other hand, it was shown that the single dose of cisplatin injection (7 mg/kg of body weight; i.p.) reduced significantly the activities of the liver PON-1 paraoxonase and arylesterase when compared with the control group (p<0.000, Table 1).

Nevertheless, pre-treatment of rats with pomegranate seed extract (300 mg/kg/day) for 15 days alleviated the cisplatin-induced decreases in PON-1 paraoxonase and arylesterase activities in the liver tissue (Table 1).

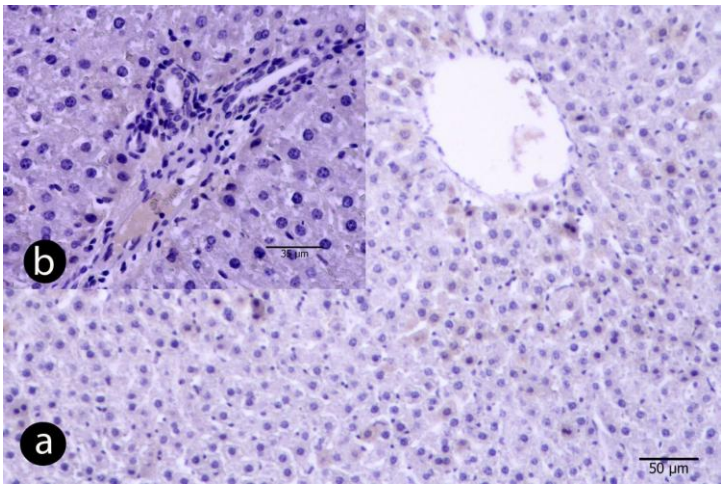

Figure 1. Bcl-x_L reactions in interlobular area (a) and around central vein (b) in control group. Streptavidin-biotin peroxidase staining.

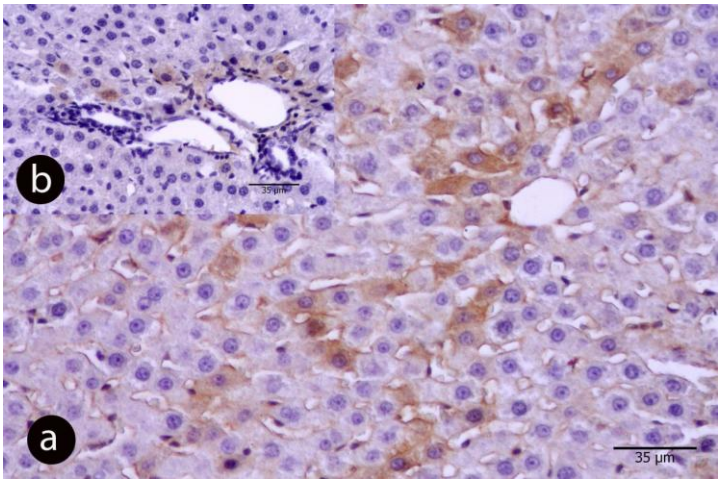

Figure 2. Bcl-xL reactions in interlobular area (a) and around central vein (b) in pomegranate seed extract group. Streptavidin-biotin peroxidase staining.

Histological results

A comparison of anti-apoptotic activity of the groups is shown in Figures 1 to 4. Based on the histological evaluation, it could be concluded that although few numbers of bcl-x_L immune positive cells were observed around the central vein in the control groups (Figure 1), these reactions were mildly observed at both the interlobular area and around the central vein in the PSE group (Figure 2). On the other hand, in the CP treated group, histological alterations were characterized with sinusoidal dilatation, hepatocellular degeneration, necrosis, and bcl-x_L immun negative hepatocyte (Figure 3). In the PSE+CP group, interestingly decreases in cytoplasmic alteration of the hepatocytes were observed when compared to the CP treated group. Anti-apoptotic activities were strongly observed in hepatocytes placed radially around the central vein in the liver tissue of PSE+CP group (Figure 4) compared with CP group. Semiquantitative analysis of bcl-x_L reactivity (anti-apoptotic) in liver was determined as follows: Control group, mild (+); PSE group, severe (+++); CP group, none or light (-/+); PSE+CP group, very strong (++++) staining immune positive reactions.

DISCUSSION

Free-radical damage and increased oxidative stress have been proposed as a mechanism of cisplatin-induced

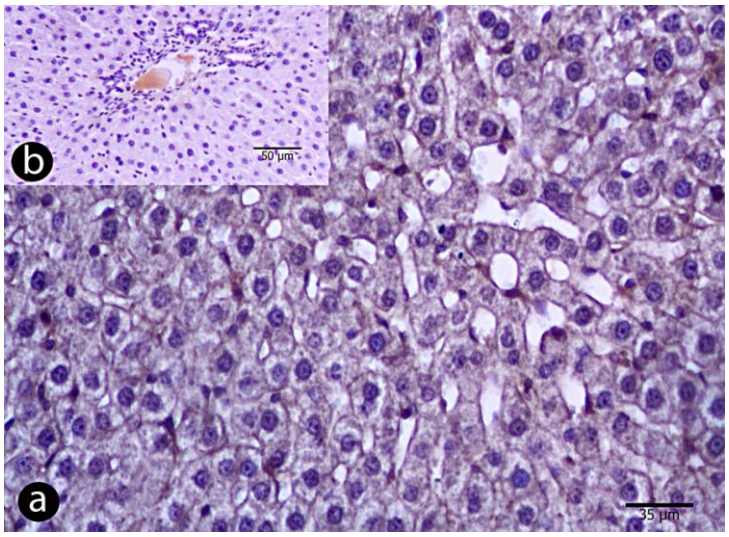

Figure 3. Bcl-xL reactions in interlobular area (a) and around central vein (b) in cisplatin control group. Streptavidin-biotin peroxidase staining.

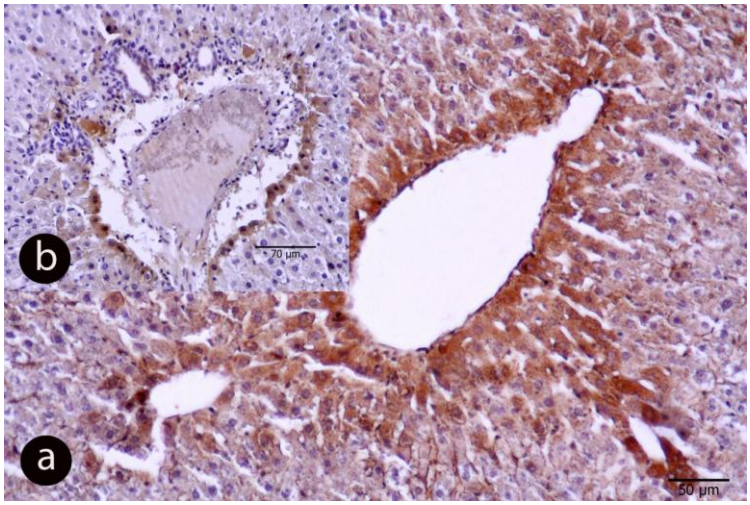

Figure 4. Bcl-xL reactions in interlobular area (a) and around central vein (b) in pomegranate seed extract+cisplatin group. Streptavidin-biotin peroxidase staining.

toxicity (Cayir et al., 2009; Cepeda et al., 2007; Martins et al., 2008). Kart et al. (2010) reported that some antioxidant molecules that are influential as scavenger or that prevent the formation of reactive oxygen species eliminate the hepatotoxicity caused by cisplatin. Therefore, we investigated whether pomegranate seed extract possesses the protective effect against the toxicity of cisplatin. For this purpose, we made both the biochemical and histopathological examination by creating an experimental rat model. In the biochemical examination, we measured serum ALT levels, PON-1 paraoxonase and arylesterase activities and MDA levels in liver tissue samples.

Cisplatin is a drug commonly used in the treatment of cancer (Gottfried et al., 2008; Turk et al., 2008a). However, especially when used at high doses, cisplatin can lead to serious side effects such as nephrotoxicity and hepatotoxicity (Jiang and Dong, 2008; Liao et al., 2008). In the animal experiment studies, it has been shown that cisplatin at a single dose of 7 mg/kg (i.p.) leads to hepatotoxicity in rats (Al-Majed, 2007). Therefore, we also have created hepatotoxicity with a

single dose injection of cisplatin (7 mg/kg, i.p.). Hepatotoxicity was shown by measuring serum ALT levels and the histological examination of the liver tissue samples after administration of cisplatin in the rats. In the present study, cisplatin led to a significant increase in the serum ALT levels when compared with the control group, and the obvious histopathological changes such as mononuclear cell infiltration, congestion and hepatocellular degeneration were observed in liver tissues of the rats. These results were consistent with those of the previous published studies of cisplatin-induced hepatotoxicity (Cayir et al., 2009; Kart et al., 2010; Yuce et al., 2007). In addition, in our study, MDA levels were increased significantly in the liver tissues from cisplatin-treated animals. This result seems to be confirmative of the literature information that increased free radical levels and resulting oxidative stress may play an important role in cisplatin-induced toxicity (Cayir et al., 2009; Martins et al., 2008).

PON-1 is a calcium-dependent esterase synthesized primarily in the liver and possesses both paraoxonase and arylesterase activities (Draganov et al., 2005). In many experimental and clinical studies, it has been revealed that PON-1 is an antioxidant enzyme and also protects HDL and LDL from oxidation (Rosenblat et al., 2006; Costa et al., 2005; Ferretti et al., 2003). In the present study, it was observed that the administration of cisplatin to rats caused a significant decrease in hepatic PON-1 paraoxonase and arylesterase activities. The decreases of these enzyme activities may be due to a direct toxic effect of cisplatin on hepatocytes or the increased free radical production (Martins et al., 2008), and namely, in cases of increased oxidative stress, the excessive amounts of free radicals can disrupt the functions of proteins by attacking specific chemical groups of proteins (Yildirim et al., 2003). PON-1 enzyme protein has three cysteine (Cys) residues in positions 42, 284 and 353, with a disulfide bond between Cys-42 and-353 and a Cys-284 as a free thiol (-SH) (Costa et al., 2005). Although Cys-284 is not essential for the hydrolytic activity of enzyme, this reactive sulfhydryl (-SH) group at position 284 is required for the tertiary structure to maintain the active-site residues in their optimal spatial arrangement (Mackness et al., 1998). Also, the free Cys-284 residue is necessary for PON-1 to be protective against LDL oxidation (Costa et al., 2005). It thus appears likely that the formation of free radicals caused by cisplatin may inhibit enzyme activity by interacting with sulfhydryl groups on PON-1 protein.

Certain antioxidants may increase PON-1 activity by preventing its oxidative inactivation. Khateeb et al. (2010) reported that pomegranate juice polyphenols stimulate PON-1 hepatic expression via PPARγ signalling cascade which result in an increased secretion of a biologically active PON-1 from the hepatocytes. In another study, whether there are beneficial effects of pomegranate juice consumption by mice on their serum PON-1 activity and macrophage PON-2 expression has been investigated (Rosenblat et al., 2010). Their data showed that pomegranate juice consumption led to a significant increase in serum PON-1 catalytic activities (36%) and macrophage PON-2 expression. Similar to the results of Khateeb et al. (2010), we observed that the consumption of pomegranate seed extract stimulates PON-1 paraoxonase and arylesterase activities in the liver tissue of rats treated with pomegranate extract. Moreover, the administration of pomegranate seed extract was observed to significantly prevent the decrease in liver paraoxonase enzyme activities caused by cisplatin. This effect is probably related to the antioxidant characteristic of pomegranate.

As we stated previously, increased free radical levels [that is, the superoxide ($O_2^{\cdot-}$), hydroxyl ($HO^{\cdot}$), perhydroxyl radicals ($HO_2^{\cdot-}$)] and/or decreased enzymatic antioxidant defense system in the cell play important roles in cisplatin toxicity (Liao et al., 2008; Cayir et al., 2009; Cepeda et al., 2007). In contrast, the pomegranates are found to be a rich source of polyphenolic compounds (flavonoids and tannins) which account for 92% of their antioxidant activities (Zahin et al., 2010), and has scavenging activity against $HO^{\cdot}$ and $O_2^{\cdot-}$ (Noda et al., 2002). Therefore, it is probable that pomegranate polyphenolic compounds inactivate free oxygen radicals such as $HO^{\cdot}$ and $O_2^{\cdot-}$ which are indeed very reactive (Yildirim et al., 2003), thus protecting the PON-1 paraoxonase and arylesterase from the harmful effects of free radicals. Cisplatin has been found to have an apoptotic effect and mitochondrial damage on liver tissue (Wetzel et al., 2001). Apoptosis is characterized by membrane budding, cell shrinkage, chromatin condensation. The caspase enzymes (initiator caspases e.g. 2, 8, 9, 10 and 12 and effector caspases e.g. 3, 6 and 7) triggers the apoptotic process, whereas bcl-x_L is generally acts anti-apoptotic, which overexpression are delay or inhibit apoptosis as well as provide a true survival advantage in cell treated with chemotherapeutic agents (Walker et al., 1997; Zhan et al., 1999).

In our previous study, CP has been found to have an apoptotic effect on proximal tubules and loop of henle in the kidney and around of the central vein in the liver (Cayir et al., 2011). In this study, anti-apoptotic cells in the liver tissue were investigated with bcl-x_L antibody. Anti-apoptotic reactions were observed throughout of the lobules and around of the central vein in the only PSE treated group and PSE pretreatment before CP injection in liver tissue, whereas bcl-x_L activity was not observed in the CP group.

Consequently, the administration of pomegranate seed extract was observed to significantly prevent the decrease in rat liver paraoxonase enzyme activities caused by cisplatin, possibly because of the antioxidant characteristic of pomegranate. Also, cisplatin-induced apoptosis may be prevented as a result of overexpression pattern of the bcl-x_L by PSE.

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