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Ameliorating effect of pomegranate juice consumption on carbon tetrachloride-induced sperm damages, lipid peroxidation, and testicular apoptosis

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Abstract

The aim of this study was to investigate whether pomegranate juice (PJ) consumption has an ameliorating effect on carbon tetrachloride (CCl_4)-induced sperm damages and testicular apoptosis associated with the oxidative stress in male rats. The study comprised of four groups (groups 1–4). Group 1 received olive oil + distilled water daily; group 2 was treated with 5 ml/kg PJ + olive oil daily; group 3 was treated with 0.25 ml/kg CCl_4 dissolved in olive oil, weekly + distilled water daily; and group 4 received weekly CCl_4 + daily PJ. All administrations were performed by gavage and maintained for 10 weeks. CCl_4 administration caused significant decreases in body and reproductive organ weights, sperm motility, concentration and testicular catalase activity, significant increases in malondialdehyde (MDA) level, and abnormal sperm rate and apoptotic index along with some histopathological damages when compared with the control group. However, significant ameliorations were observed in absolute weights of testis and epididymis, all sperm quality parameters, MDA level, apoptotic index, and testicular histopathological structure following the administration of CCl_4 together with PJ when compared with group given CCl_4 only. In conclusion, PJ consumption ameliorates the CCl_4-induced damages in male reproductive organs and cells by decreasing the lipid peroxidation.

Keywords

Apoptosis, carbon tetrachloride, lipid peroxidation, pomegranate juice, sperm, testis

Introduction

Carbon tetrachloride (CCl_4), a colorless toxic substance, is rapidly absorbed by any route of exposure in humans and animals after it is released into the environment predominantly through direct emissions to air, with lower amounts discharged to soil and water. Once absorbed, it is widely distributed among tissues, especially those with high lipid content, reaching peak concentrations within <1–6 h, depending on the exposure concentration or dose. (U.S. EPA. IRIS, 2010). CCl_4 has widely been used as *in vivo* and/or *in vitro* study model to observe free radicals-induced injuries in different organs including mainly liver (Karakus et al., 2011; Nogueira et al., 2009) and also kidney, testis (Fadhel and Amran, 2002; Manjrekar et al., 2008), lung (Abraham et al., 1999; Ögetürk et al., 2009) and brain (Soliman and Fahmy, 2011). It has been reported that cytochrome P450 (CYP)-mediated excessive generation of free radicals is responsible for CCl_4-induced

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hepatotoxicity. CYP isozymes activate CCl_4 into its active metabolite, trichloromethyl radical in the liver (Abraham et al., 1999; Sheweita et al., 2001). The trichloromethyl radical then reacts with oxygen to form the highly toxic reactive trichloromethyl peroxyl radical. The free radicals subsequently attack the membrane lipids, especially polyunsaturated fatty acids (PUFAs), leading to breakdown of membrane structure and disruption of cell energy processes and protein synthesis leading to the progression of liver damage (Nogueira et al., 2009; Weber et al., 2003).

Spermatozoa require a high PUFA content to provide the plasma membrane with the fluidity essential at fertilization. However, this makes spermatozoa particularly vulnerable to attack by reactive oxygen species (ROS) (Wathes et al., 2007). In addition, as the testis, prostate (Jiang et al., 1998), epididymis (Hudson et al., 2001), and germ cells (Liu et al., 2007) contain CYP isozymes, it is possible that CCl_4 causes oxidative damage to lipids of these tissues and cells (Abraham et al., 1999). Acute or chronic CCl_4 administration has been reported to cause increments in testicular tissue lipid peroxidation (LPO) level (Abraham et al., 1999; Fadhel and Amran, 2002; Khan, 2012; Khan and Ahmed, 2009; Soliman and Fahmy, 2011; Yüce et al., 2013), sperm shape abnormalities (Abdou et al., 2012; Yüce et al., 2013) and testicular tissue DNA fragmentation (Abdou et al., 2012; Khan, 2012; Yüce et al., 2013), reductions in weights of body and testes (Castilla-Cortazar et al., 2004; Manjrekar et al., 2008; Khan and Ahmed, 2009; Yüce et al., 2013), antioxidant enzymes (Khan, 2012; Khan and Ahmed, 2009; Soliman and Fahmy, 2011; Yüce et al., 2013), sperm count and motility (Khan, 2012; Yüce et al., 2013), degeneration in testicular histologic structure (Castilla-Cortazar et al., 2004; Horn et al., 2006; Kalla and Bansal, 1975; Khan and Ahmed, 2009; Yüce et al., 2013), disturbances in steroid and gonadotropin hormones (Castilla-Cortazar et al., 2004; Khan, 2012; Khan and Ahmed, 2009).

Antioxidants are compounds that scavenge and suppress the formation of ROS and LPO. Hence, the application of ROS scavengers is likely to ameliorate the stress-induced damages in testis and sperm function (Vernet et al., 2004). For this purpose, some herbal antioxidants were used to prevent CCl_4-induced testicular oxidative stress (Fadhel and Amran, 2002; Manjrekar et al., 2008; Khan, 2012; Khan and Ahmed, 2009; Soliman and Fahmy, 2011; Yüce et al., 2013), hormonal disturbances (Khan, 2012; Khan and Ahmed, 2009), deteriorated sperm quality and testicular tissue DNA fragmentation (Khan, 2012; Yüce et al., 2013), and some testicular histopathological lesions (Khan and Ahmed, 2009; Manjrekar et al., 2008; Yüce et al., 2013) in rats. Pomegranate (*Punica granatum* L.) is an ancient fruit that is widely consumed as fresh fruit and juice. Pomegranate juice (PJ) has been reported to be a rich source of vitamin C, minerals, and polyphenols (Viuda-Martos et al., 2010). The high antioxidant activity of PJ has been attributed to its high polyphenolic content (Johanningsmeier and Harris, 2011). PJ or pomegranate extracts improve sperm count, motility, and abnormal sperm rate in nonstressed healthy laboratory animals (Amini Rad et al., 2009; Türk et al., 2008) and also in oxidatively stressed animals (Al-Daraji, 2012; El Ghazzawy et al., 2011; Leiva et al., 2011). On the other hand, it has been demonstrated in a study that pomegranate peels and seeds decrease CCl_4-induced sperm shape abnormalities (Abdou et al., 2012). However, there is no evidence regarding ameliorating effect of PJ on CCl_4-induced damages on other spermatological parameters including sperm count and motility and on testicular apoptotic cell index. Therefore, this study was conducted to investigate whether PJ has any ameliorating effect on CCl_4-induced negative changes in sperm quality, testicular apoptosis, and histopathological lesions associated with the oxidative stress.

Materials and methods

PJ and chemicals

Pasteurized PJ (100% pure, pasteurized juice, 250 ml, Elite Natural Beverage Co., Ankara, Turkey) was purchased from a local store. The other chemicals were purchased from Sigma-Aldrich Chemical Co. (St Louis, Missouri, USA).

Animals and experimental design

The experimental protocols were approved by the local Committees for using Animals of Firat University (Elazig, Turkey). Animal care and experimental protocols complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. A total of 28 healthy adult male Wistar albino rats, aged 5 months, were obtained from Firat University Experimental Research Centre, Elazig, Turkey, and maintained therein during the study. The animals were housed in polycarbonate cages in a room with a 12 h day–night cycle at a temperature of 24 $\pm$ 3°C and humidity of 45–65%. During the whole experimental period, animals were fed with a balanced commercial

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diet (Elazig Food Company, Elazig, Turkey) and fresh drinking water was given *ad libitum*.

The rats were randomly divided into four groups (groups 1–4); each containing seven rats. Group 1 received 0.5 ml pure olive oil + 1.5 ml distilled water daily by gavage and they served as control. Group 2 (group PJ) was administered with 5 ml/kg PJ (approximately 1.5 ml volume for each rat) + 0.5 ml pure olive oil daily by gavage. The rats in group 3 were treated with 0.25 ml/kg CCl_4, which dissolved in 0.5 ml pure olive oil weekly + 1.5 ml distilled water daily (group CCl_4). The rats in group 4 received weekly CCl_4 and daily PJ (Group CCl_4 + PJ). All administrations were maintained for 10 weeks. Olive oil was used as vehicle because CCl_4 is an oil-dissolved chemical. The doses of CCl_4 (Horn et al., 2006; Yüce et al., 2013) and PJ (Türk et al., 2008) given to rats in this study, generally used for long-term studies, were selected based on the previous reports. The spermatogenic cycle, including spermatocytogenesis, meiosis, and spermiogenesis, is 48–52 days (Bennett and Vickery, 1970), and the epididymal transit of spermatozoa is approximately 1 week (Kempinas et al., 1998) in rats; the treatment period used herein was set at 10 weeks to achieve a maximum effect.

Sample collection and homogenate preparation

The rats were killed using ether anesthesia at the end of 10th week. Testes, epididymides, seminal vesicles, and ventral prostate were removed and cleared from adhering connective tissue and weighed. One of the testis samples was fixed in Bouin's solution for histopathological examination. The other testes samples were stored at −20°C for biochemical analyses. Testes were taken from −20°C freezer and immediately transferred to the cold glass tubes. Then, the testes were diluted with a ninefold volume of phosphate-buffered saline (PBS; pH 7.4). For the enzymatic analyses, testes were minced in a glass and homogenized by a Teflon–glass homogenizator for 3 min in cold physiological saline on ice (Türk et al., 2011).

Testicular tissue LPO level and antioxidant enzyme activities

All analyses were performed with the aid of a spectrophotometer (2R/UVultraviolet–visible, Shimadzu, Tokyo, Japan). LPO level was measured according to the concentration of thiobarbituric acid reactive substances (TBARS), and the amount of malondialdehyde (MDA) produced was used as an index of LPO. The MDA level at 532 nm was expressed in nanomole per gram protein (Placer et al., 1966).

Reduced glutathione (rGSH) level was measured using the method described by Sedlak and Lindsay (1968). The level of rGSH at 412 nm was expressed as nanomole per gram protein. Glutathione peroxidase (GSH-Px, EC 1.11.1.9) activity was determined according to the method described by Lawrence and Burk (1976). The GSH-Px activity at 340 nm was expressed in international unit per gram protein. Catalase (CAT, EC 1.11.1.6) activity was determined by measuring the decomposition of hydrogen peroxide (H_2O_2) at 240 nm and was expressed in k per gram protein, where k is the first-order rate constant (Aebi, 1983). Protein concentration was determined using the method described by Lowry et al. (1951).

Sperm analyses

All sperm analyses were made using the methods reported in the study by Türk et al. (2008). The sperm concentration in the right cauda epididymal tissue was determined with a hemocytometer. Freshly isolated left cauda epididymal tissue was used for the motility analysis of sperm. The percentage of sperm motility was evaluated using a light microscope with a heated stage. To determine the percentage of morphologically abnormal spermatozoa; the slides stained with eosin–nigrosin (1.67% eosin, 10% nigrosin, and 0.1 M of sodium citrate) were prepared and then viewed under a light microscope at 400× magnification. A total of 300 spermatozoa were examined in each slide (2100 cells in each group), and the head, tail, and total abnormality rates of spermatozoa were expressed in percentage.

Histopathological examination

Testis tissues were fixed in Bouin's solution for 48 h, dehydrated through graded concentrations of ethanol, embedded in paraffin wax, sectioned at 5 μm thickness, and stained with Mayer's hematoxylin and eosin. A total of 25 seminiferous tubules (ST) were randomly examined in each section, their diameters and germinal cell layer thicknesses (GCLT; from the basal membrane toward the lumen of the tubule) were measured by an ocular micrometer in a light microscope and the mean size of ST and GCLT were calculated. Johnsen's testicular scoring (Johnsen, 1970) was performed for the control and treated groups. In total, 25 ST from each section were evaluated, and a

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score between 1 (*very poor*) and 10 (*excellent*) was given to each tubule according to Johnsen's criteria. The degree of damages was graded as follows: *mild* (+), *moderate* (++), and *severe* (+++).

Testicular apoptotic cell index

The apoptotic germ cells were defined by terminal deoxynucleotidyl transferase (TdT)-mediated 2′-deoxyuridine 5′-triphosphate (dUTP) nick end-labeling (TUNEL) assay with the ApopTag Peroxidase in Situ Apoptosis Detection Kit (Chemicon, Temecula, California, USA) according to the manufacturer's instructions. The fixed testicular tissues in Bouin's solution were embedded in paraffin and sectioned at 4 μm thickness. The paraffin sections were deparaffinized in xylene, dehydrated through graded alcohol, and washed in PBS. The sections were treated with 20 mg/ml proteinase K for 5 min, followed by treatment with 3% H_2O_2 for 5 min to inhibit endogenous peroxidase. After rewashing with PBS, sections were then incubated with the TUNEL reaction mixture containing TdTenzyme and digoxigenin–11-dUTP at 37°C for 1 h in humidified chamber, and then stop/wash buffer was applied for 30 min at 37°C. Sections were visualized with 3-amino-9-etilcarbazole substrate. Negative controls were performed using distilled water in the place of the TdT enzyme. Finally, sections were counterstained with Mayer's hematoxylin, rinsed in tap water, and mounted with glycerol. TUNEL-positive apoptotic cell index was calculated using the following equation

$$\text{TUNEL} - \text{positive apoptotic cell index}(\%) = \frac{\text{Total apoptotic cellcount in 25 ST}}{\text{Total germinal cell count in 25 ST}} \times 100$$

Statistical analysis

Data are presented as the mean ± SEM. The degree of significance was set at $p < 0.05$. It was determined that raw data showed normal distribution according to Shapiro–Wilk normality test. Based on the normality test, one-way analysis of variance and *post hoc* Tukey's honestly significant difference test were used to determine the differences between the groups with respect to all parameters. All the analyses were carried out using the Statistical Package for Social Sciences (SPSS)/PC software program (Version 15.0; SPSS, Chicago, Illinois, USA).

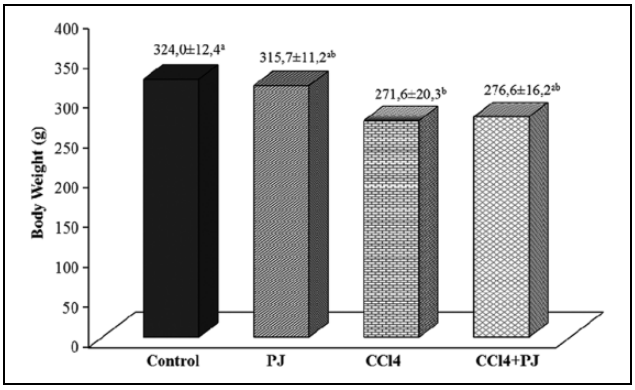

Figure 1. Mean ± SEM values of body weight in different treatment groups (control, PJ, CCl_4, and CCl_4 + PJ). The mean values having different superscripts (a and b; $p < 0.05$) in each group significantly differ from each other. PJ: pomegranate juice; CCl_4: carbon tetrachloride.

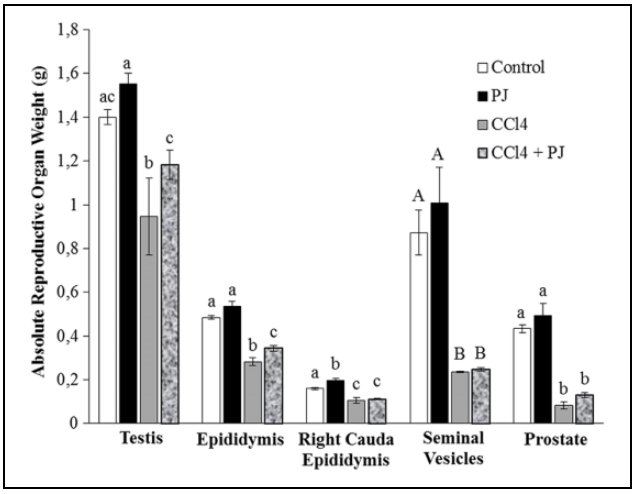

Figure 2. Mean ± SEM values of absolute reproductive organ weights in different treatment groups (control, PJ, CCl_4, and CCl_4 + PJ). The mean values having different superscripts (a, b, and c; $p < 0.001$, A and B; $p < 0.01$) in each group significantly differ from each other. PJ: pomegranate juice; CCl_4: carbon tetrachloride.

Results

Body and reproductive organ weights

Administration of CCl_4 caused a significant ($p < 0.05$) decrease in the final body weight when compared with the control group. Consumption of PJ by CCl_4-treated rats provided insignificant increase in body weight in comparison with the CCl_4 group only (Figure 1). Absolute and relative reproductive organ weights are presented in Figures 2 and 3, respectively. Only PJ administration significantly increased the absolute

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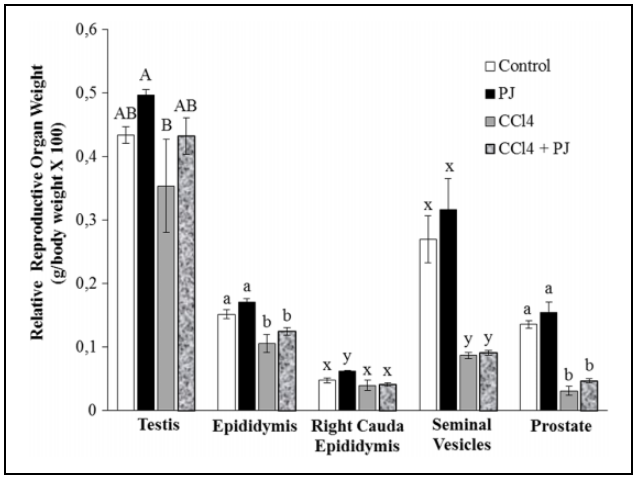

Figure 3. Mean ± SEM values of relative reproductive organ weights in different treatment groups (control, PJ, CCl_4, and CCl_4 + PJ). The mean values having different superscripts (A and B; $p < 0.05$, a and b; $p < 0.001$, x and y; $p < 0.01$) in each group significantly differ from each other. PJ: pomegranate juice; CCl_4: carbon tetrachloride.

($p < 0.001$) and relative ($p < 0.01$) weights of the right cauda epididymis in comparison with the control group. Administration of CCl_4 caused significant reductions in absolute weights of testis ($p < 0.001$), epididymis ($p < 0.001$), right cauda epididymis ($p < 0.001$), seminal vesicles ($p < 0.01$), and prostate ($p < 0.001$) and relative weights of epididymis ($p < 0.001$), seminal vesicles ($p < 0.01$), and prostate ($p < 0.001$). However, significant increases ($p < 0.001$) were determined in absolute weights of testis and epididymis in CCl_4 + PJ group when compared with CCl_4 group only.

Testicular tissue LPO level and antioxidant enzyme activities

Testicular tissue LPO, demonstrated as MDA, and GSH level, GSH-Px and CAT activities of all the groups are given in Table 1. Only PJ administration tended to decrease the MDA level and to increase the rGSH level, GSH-Px, and CAT activities, but these improvements did not reach the statistical significance when compared with the control group. Only CCl_4 administration caused significant ($p < 0.001$) increase in MDA level and significant ($p < 0.01$) decrease in CAT activity when compared with the control group. In addition, CCl_4 tended to decrease rGSH level and GSH-Px activity, but these decreases did not reach the statistical significance when compared with the control group. However, PJ administration to CCl_4-treated rats significantly ($p < 0.001$) decreased the CCl_4-induced increment in MDA level. Although the increase observed in CAT activity after PJ administration to CCl_4-treated rats is not statistically significant in comparison with the CCl_4 group, CAT activity of CCl_4 + PJ group was comparable with that of the control group.

Sperm parameters

Epididymal sperm concentration, sperm motility, and abnormal sperm rate in all groups are presented in Table 2. Only PJ administration significantly ($p < 0.001$) increased sperm motility and concentration in comparison with the control group. Significant decreases ($p < 0.001$) in sperm motility and concentration and significant increases ($p < 0.001$) in head, tail, and total abnormal sperm rates were observed in CCl_4 group when compared with the control group. However, PJ consumption by rats treated with CCl_4 provided significant ($p < 0.001$) ameliorations in all sperm parameters when compared with the CCl_4 group only.

Testicular histopathological lesions and apoptotic cell index

No histopathological lesions (Table 3) were observed in testicular tissues of control (Figure 4(a)) and PJ (Figure 4(b)) groups. The histopathological changes such as necrosis, degeneration, desquamation, disorganization, and reduction in germinal cells, atrophy in tubules, thickening in basal membrane, interstitial edema and congestion, multinuclear syncytial cell formation, and spermatogenic arrest were observed in CCl_4 (Figure 4(c)) and CCl_4 + PJ (Figure 4(d)) groups. Almost all ST in testes of CCl_4 group contained a great number of spermatogonia, but with a very few numbers of spermatocytes and spermatids when compared with the control group. However, increases in the number of spermatocytes and spermatids as well as spermatogonia were observed in ST of CCl_4 + PJ group in comparison with the CCl_4 group. In addition, the degree of lesions was significantly ($p < 0.001$) worse in CCl_4 group than CCl_4 + PJ group (Table 3). Significant ($p < 0.001$) decreases in diameters of ST, GCLT, and Johnsen's testicular score were determined in CCl_4 group as compared to the control group. However, PJ administration to CCl_4-treated animals significantly ($p < 0.001$) ameliorated the CCl_4-induced damages in these parameters (Table 4).

Figure 5 illustrates apoptosis, demonstrated by TUNEL-staining, in the testis of control and treated

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Table 1. Mean ± SEM values of MDA, rGSH levels, GSH-Px, and CAT activities in different treatment groups.[a]

	Oxidative stress markers			
Groups	MDA (nmol/g protein)	rGSH(nmol/g protein)	GSH-Px (IU/g protein)	CAT (*k*/g protein)
Control	7.72 ± 0.34[b]	5.79 ± 0.46	1.08 ± 0.25	48.04 ± 3.75[A]
PJ	6.33 ± 1.05[b]	6.03 ± 0.74	1.69 ± 0.34	63.78 ± 11.66[A]
CCl_4	17.34 ± 0.72[c]	4.21 ± 0.38	0.65 ± 0.14	13.05 ± 1.88[B]
CCl_4+PJ	7.03 ± 1.68[b]	5.67 ± 0.32	0.96 ± 0.29	38.31 ± 5.14[A,B]

MDA: malondialdehyde; rGSH: reduced glutathione; GSH-Px: glutathione peroxidase; CAT: catalase; PJ: pomegranate juice; CCl_4: carbon tetrachloride.
[a]The mean values having different superscripts (b and c: $p < 0.001$; A and B: $p < 0.01$) within the same column significantly differ from each other.

Table 2. Mean ± SEM values of sperm parameters in different treatment groups.[a]

	Parameters				
			Abnormal sperm rate (%)		
Groups	Sperm motility (%)	Epididymal sperm concentration (million/right cauda epididymis)	Head	Tail	Total
Control	74.64 ± 1.99[b]	87.40 ± 4.50[b]	3.80 ± 0.91[b]	7.00 ± 1.22[b,c]	10.80 ± 1.98[b]
PJ	91.66 ± 0.89[d]	117.42 ± 3.70[d]	3.42 ± 0.42[b]	5.00 ± 0.65[b]	8.42 ± 0.99[b]
CCl_4	22.20 ± 6.17[c]	29.66 ± 13.16[c]	18.33 ± 1.45[d]	21.33 ± 4.63[d]	39.66 ± 6.06[d]
CCl_4 + PJ	51.28 ± 2.00[e]	52.60 ± 9.90[e]	7.40 ± 0.50[c]	10.80 ± 0.80[c]	18.20 ± 2.58[c]

PJ: pomegranate juice; CCl_4: carbon tetrachloride.
[a]The mean values having different superscripts (b, c, d, and e: $p < 0.001$) within the same column significantly differ from each other.

groups. The apoptotic cell index of CCl_4 group was significantly ($p < 0.001$) higher than that of the control group. However, a significant ($p < 0.001$) decrease was observed in apoptotic cell index of CCl_4 + PJ group compared with that of the CCl_4 group only (Table 4).

Discussion

Male reproductive dysfunction constitutes half of the infertility in humans and animals. Numerous toxic substance-induced disruptions in oxidant/antioxidant balance of male reproductive system are one of the important factors that cause the male infertility. This study was aimed to investigate the toxic effects of CCl_4 and also improvement effects of PJ through oxidative stress mechanism on reproductive system of adult male rats; we examined the changes in reproductive organ weights, sperm parameters, testicular tissue oxidative stress markers, testicular histologic structure, and apoptotic germ cells.

Androgens stimulate the growth by inducing the protein synthesis (Fernandes et al., 2007), but increase in free radicals causes oxidation of proteins together with lipids in tissues (Abraham et al., 1999; Khan, 2012). In addition, permanent androgenic stimulation is necessary for normal growth and functions of testes, epididymides, and accessory sex organs (Klinefelter and Hess, 1998). Therefore, disturbances in the synthesis of androgens and proteins can cause negative changes in body weight and especially in the reproductive organ weights (Fernandes et al., 2007). It has been reported that CCl_4 administration results in reduced body and testis weights (Castilla-Cortazar et al., 2004; Manjrekar et al., 2008; Khan and Ahmed, 2009; Yüce et al., 2013) and decreased testosterone level (Khan, 2012; Khan and Ahmed, 2009). In the present study, CCl_4 caused significant decreases in body weight and also weights of testes, epididymides, and accessory sex glands. These decreases in the weights of body and reproductive organ weights observed in the present study may possibly be explained by CCl_4-induced decreased testosterone concentration (Khan and Ahmed, 2009) in conjunction with protein oxidation (Abraham et al., 1999; Khan, 2012) due to the excessive generation of free radicals.

Oxidative stress is an imbalance between the production of high ROS and their efficient removal by

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Table 3. The degree of some pathological lesions in testicular tissues of different treatment groups.[a]

Lesions	Control	PJ	CCl_4	CCl_4 + PJ
Necrosis in germinal cells	ND	ND	2.48 ± 0.19[b]	1.00 ± 0.31[c]
Atrophy in seminiferous tubules	ND	ND	2.20 ± 0.10[b]	1.57 ± 0.20[c]
Thickening in tubule basal membrane	ND	ND	2.13 ± 0.22[b]	1.29 ± 0.18[c]
Degeneration in germinal cells	ND	ND	2.75 ± 0.16[b]	1.29 ± 0.18[c]
Desquamation in germinal cells	ND	ND	2.57 ± 0.30[b]	1.00 ± 0.00[c]
Reduction in germinal cell counts	ND	ND	3.00 ± 0.05[b]	1.14 ± 0.14[c]
Disorganization in germinal cells	ND	ND	3.00 ± 0.09[b]	2.00 ± 0.00[c]
Vacuolization in germinal cells	ND	ND	1.47 ± 0.21[b]	1.14 ± 0.14[b]
Interstitial edema and congestion	ND	ND	2.66 ± 0.10[b]	1.29 ± 0.18[c]
Multinucleated syncytial cell formations	ND	ND	3.00 ± 0.18[b]	0.57 ± 0.30[c]
Spermatogenic arrest	ND	ND	2.90 ± 0.14[b]	1.14 ± 0.26[c]

ND: not detected; PJ: pomegranate juice; CCl_4: carbon tetrachloride.
[b]Different from both control and PJ groups ($p < 0.001$).
[c]Different from CCl_4 group ($p < 0.001$).

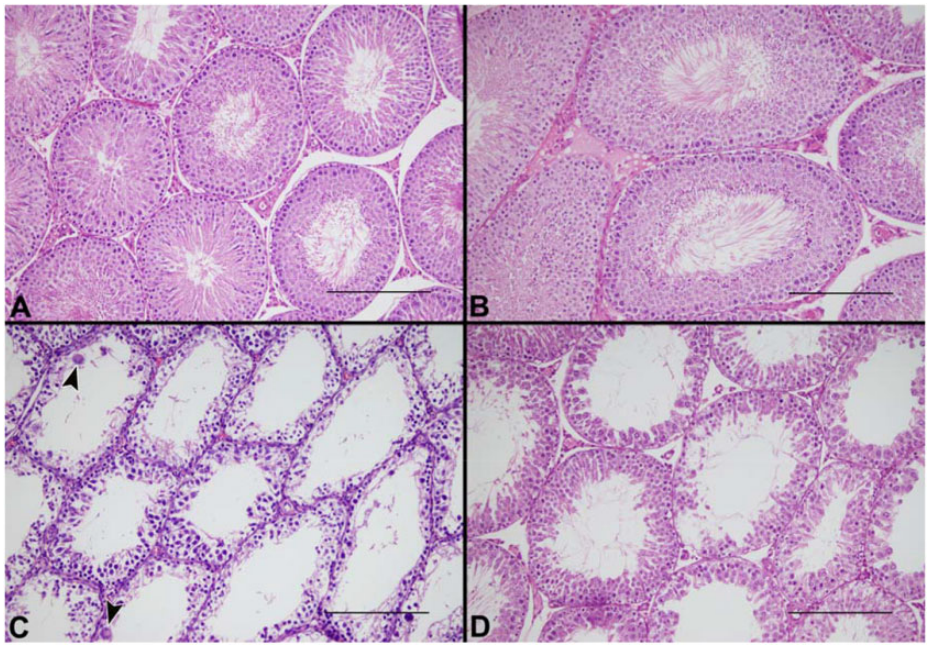

Figure 4. Representative photomicrographs of histopathological structure of testis in different treatment groups (control, PJ, CCl_4, and CCl_4 + PJ, calibration bar = 200 μm). (a) Hematoxylin and eosin staining in control group. (b) Hematoxylin and eosin staining in PJ-treated group. (c) Hematoxylin and eosin staining in CCl_4-treated group (arrows show multinuclear syncytial cells). (d) Hematoxylin and eosin staining in CCl_4 + PJ-treated group. PJ: pomegranate juice; CCl_4: carbon tetrachloride.

antioxidant systems. Superoxide anion $\left(O_2^-\right)$, H_2O_2, and the hydroxyl ion ($^{\bullet}OH$) are the most prominent ROS. They can be produced in large amounts by spermatozoa, mitochondria, and a variety of enzymes including the xanthine- and nicotinamide adenine dinucleotide phosphate oxidases, and by CYP isozymes in the testis under pathologic conditions. O_2^- is largely generated as a result of redox reactions within the mitochondria, but in most situations O_2^- is quickly converted to H_2O_2 by the superoxide dismutase. H_2O_2 can undergo reactions with heavy metals and $^{\bullet}OH$ or can be detoxified through the GSH/GSH-Px pathway to yield water and rGSH. H_2O_2 can also be reduced by CAT to produce oxygen and water. Free radicals have high affinity to cell membrane lipids, especially PUFAs, leading to tissue damage due to the LPO

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Table 4. Mean ± SEM values of diameters of ST, GCLT, Johnsen testicular score, and TUNEL positive apoptotic cell index in different treatment groups.[a]

Groups	Variables			
	Diameter of ST (μm)	GCLT (μm)	Johnsen testicular score (1–10)	TUNEL positive apoptotic cell index (%)
Control	249.97 ± 1.68[b]	102.07 ± 1.13[b]	9.67 ± 0.21[b]	0.93 ± 0.16[b]
PJ	261.48 ± 1.84[c]	103.35 ± 0.80[b]	10.00 ± 0.00[b]	0.90 ± 0.21[b]
CCl_4	183.78 ± 2.35[d]	43.27 ± 1.25[c]	4.75 ± 0.29[c]	4.87 ± 0.32[c]
CCl_4 + PJ	231.48 ± 1.68[e]	75.42 ± 1.05[d]	7.00 ± 2.92[d]	3.32 ± 0.11[d]

STs: seminiferous tubules; GCLT: germinal cell layer thickness; PJ: pomegranate juice; CCl_4: carbon tetrachloride
[a]The mean values having different superscripts (b, c, d, and e: $p < 0.001$) within the same column significantly differ from each other.

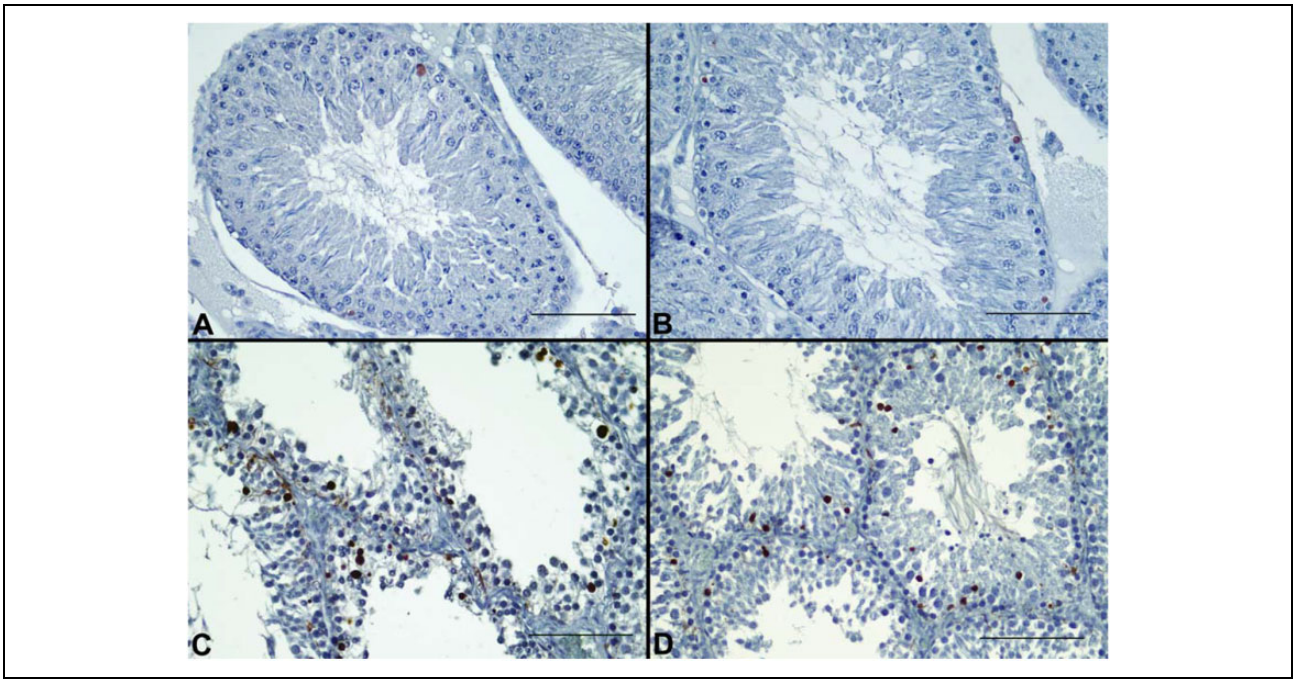

Figure 5. Representative photomicrographs of apoptotic cells by TUNEL method in the testis of different treatment groups (control, PJ, CCl_4, and CCl_4 + PJ, calibration bar = 100 μm). (a) TUNEL-staining in control group. (b) TUNEL staining in PJ-treated group. (c) TUNEL staining in CCl_4-treated group (significant reduction in germinal cells and brown–red stained cells are the apoptotic ones. Significant increase is seen in the apoptotic cell index calculated by dividing total apoptotic cell number into total germinal cell number in 25 STs). (d) TUNEL staining in CCl_4 + PJ-treated group (significant increment in germinal cells and brown–red stained cells are the apoptotic ones. Significant decrease is seen in the apoptotic cell index calculated by dividing total apoptotic cell number into total germinal cell number in 25 STs). TUNEL: terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick end-labeling; PJ: pomegranate juice; CCl_4: carbon tetrachloride; STs: seminiferous tubules.

(Aitken and Roman, 2008; Turner and Lysiak, 2008). It has been reported that CCl_4 causes increases in MDA or TBARs levels, which are by-products of LPO (Abraham et al., 1999; Castilla-Cortazar et al., 2004; Fadhel and Amran, 2002; Khan, 2012; Khan and Ahmed, 2009; Soliman and Fahmy, 2011; Yüce et al., 2013) and decreases in enzymatic and nonenzymatic antioxidants in testicular tissue (Khan, 2012; Khan and Ahmed, 2009; Soliman and Fahmy, 2011). CCl_4 administration caused significant increase in testicular MDA level and significant decrease in testicular CAT activity and also nonsignificant decrease in rGSH level and GSH-Px activity in this study. In the present study, the CCl_4-induced oxidative damage in testes may depend on the increased free radicals mediated by CYP activity, which was also identified in testes (Jiang et al., 1998).

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Spermatogenesis is an extremely active replicative process and has high rates of cell division. This process leads to high rates of mitochondrial oxygen consumption by spermatogenic cells and the germinal epithelium. However, the poor vascularization of the testes means that oxygen tensions in this tissue are low and that competition for this vital element within the testes is extremely intense. Despite the low oxygen tensions that characterize the testicular microenvironment, spermatozoa and other cells within the testis remain vulnerable to oxidative stress due to the abundance of high PUFAs and the presence of potential ROS-generating systems (Aitken and Roman, 2008). On the other hand, after spermatozoa are passively left from the testis, they acquire functional competence by some modifications in its plasma membrane including remodeling, acquisition, and shedding of sperm surface proteins during epididymal transit. Therefore, spermatozoa are also vulnerable to oxidative damage during the posttesticular phase due to the maturational changes in sperm plasma membrane (Vernet et al., 2004). Thus, excessive generation of free radicals in pathologic conditions can induce the LPO by oxidative breakdown of PUFAs in the membranes of cells. Obviously, peroxidation of sperm lipids destroys the structure of lipid matrix in the membranes of spermatozoa, and it is associated with rapid loss of intracellular adenosine triphosphate leading to axonemal damage, decreased sperm viability, and increased mid-piece morphological defects, and even it completely inhibits spermatogenesis in extreme cases (Aitken and Roman, 2008; Turner and Lysiak, 2008). In the present study, significant decreases in epididymal sperm concentration and motility and significant increases in head, tail, and total abnormal sperm rates were observed in CCl_4 group when compared with the control group. These findings are in agreement with the earlier reports that demonstrated a reduced sperm count and sperm motility (Khan, 2012; Yüce et al., 2013) and also an increased sperm shape abnormality (Abdou et al., 2012; Khan, 2012; Yüce et al., 2013) in CCl_4-treated rats. Increased LPO and decreased antioxidant enzyme activity, as evidenced by increased MDA level and decreased CAT activity in this study, may be responsible for impaired sperm quality observed in CCl_4-treated rats.

It has been reported that long-term CCl_4 administration (from 20 days to 16 weeks) leads to severe damage to the spermatogenic cycle such as exfoliation in the germinal epithelium, depletion and degeneration in germ cells, shrinkage in the tubules, vacuolization in germinal epithelium, and meiotic arrest (Horn et al., 2006; Kalla and Bansal, 1975; Khan, 2012; Khan and Ahmed, 2009; Yüce et al., 2013). However, short-term administration (10–15 days) of CCl_4 has no significant adverse effect on testicular structure (Castilla-Cortazar et al., 2004; Kalla and Bansal, 1975), but it alters hematotesticular barrier (Castilla-Cortazar et al., 2004). Similarly, prominent histopathological damages such as necrosis, degeneration, desquamation, disorganization, reduction in germinal cells, spermatogenic arrest, and significant decreases in diameters of ST, GCLT, and Johnsen's testicular score were determined in CCl_4 group as compared to the control group in this study. Apoptosis is known to be a programmed cell death for controlling the spermatogonial population within the testis. However, increased rate of apoptotic germ cells in pathologic conditions disrupts this program leading to excessive cell death (Blanco-Rodriguez, 1998). Excessive generation of free radicals-induced DNA damage results in increased testicular apoptotic germ cell (Maheshwari et al., 2009). It has been reported that long-term CCl_4 administration causes testicular DNA damage (Khan, 2012; Yüce et al., 2013). The apoptotic cell index of CCl_4 group was found to be significantly higher than the control group in the present study. Increased LPO level and decreased antioxidant activity induced by CCl_4 administration may possibly cause the testicular histopathological damages and the increase in testicular apoptotic cell index.

Natural antioxidants are found in various plant products such as fruits, leaves, juices, seeds, and oils. They have attracted special interest because of their ability to decrease the xenobiotics- and the foreign chemicals-induced increase in free radicals, which can cause various degenerative diseases. Pomegranate, in addition to ancient historical uses, has been used in several systems of medicine for a variety of ailments. PJ is a polyphenol-rich juice with high antioxidant capacity (Johanningsmeier and Harris, 2011; Viuda-Martos et al., 2010). It has been reported that oral consumption of PJ provides significant reduction in testicular tissue MDA level and significant increments in rGSH level, GSH-Px, and CAT activities (Türk et al., 2008; Yüce and Aksakal, 2007) and also significantly improves sperm count, motility, and abnormal sperm rate in nonstressed healthy laboratory animals (Amini Rad et al., 2009; Türk et al., 2008). In addition, *in vitro* PJ supplementation to semen of rooster improves sperm motility, viability, and acrosomal integrity during cool storage (Al-Daraji, 2012). Similarly, PJ or pomegranate extracts have been reported

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to ameliorate the damages in sperm quality associated with oxidative stress induced by lead acetate (Leiva et al., 2011) and bisphenol-A (El Ghazzawy et al., 2011). On the other hand, it has been demonstrated so far in only one study that pomegranate peels and seeds decrease the CCl_4-induced sperm shape abnormalities (Abdou et al., 2012). However, to our knowledge, till now, no comprehensive scientific study has been performed on ameliorating effect of PJ on testicular oxidative stress, sperm damages, histopathological lesions, and testicular apoptosis induced by CCl_4. Therefore, this is the first comprehensive report regarding the ameliorating effect of PJ on CCl_4-induced reproductive dysfunction in males. In this study, long-term PJ administration to CCl_4-treated rats significantly decreased the increments in testicular LPO level, abnormal sperm rates, testicular histopathological lesions, and testicular apoptotic cell index and significantly increased the reductions in CAT activity, absolute weights of testis and epididymis, sperm concentration, and motility when compared with the CCl_4 group. Besides, it has been reported that repeated doses of antioxidants could reduce the toxic effects exerted by CCl_4 upon the liver, and probably other organs, through inhibition of CYP system that activates CCl_4 into its active metabolite, trichloromethyl radical (Sheweita et al., 2001). PJ, a potent antioxidant, has been demonstrated to inhibit CYP activity/expression (Faria et al., 2007). The ameliorations observed in these parameters may possibly be related to the potent antioxidant and radical scavenging activity of PJ (Yüce and Aksakal, 2007; Türk et al., 2008) and also its inhibitory effect on CYP activity/expression (Faria et al., 2007).

In conclusion, the findings of the present study clearly suggest that PJ has ameliorating effect on CCl_4-induced damages in male reproductive system. This ameliorating effect of PJ seems to be closely associated with the scavenging of free radicals and suppressing of LPO due to the inhibition of CYP activity.

Conflict of interest

The authors declared no conflicts of interest.

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