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Original Research

Effect of pomegranate (*Punica granatum* L.) juice on kidney, liver, heart and testis histopathological changes, and the tissues lipid peroxidation and antioxidant status in lead acetate-treated rats

D. S. Aksu[1*], Y. S. Sağlam[2], S. Yildirim[2], T. Aksu[3]

[1] Department of Physiology, Faculty of Veterinary Medicine, Yuzuncu Yıl University, 65080,Van, Turkey
[2] Department of Pathology, Faculty of Veterinary Medicine, Ataturk University, 25100, Erzurum, Turkey
[3] Department of Animal Science, Faculty of Veterinary Medicine, Yuzuncu Yıl University, 65080, Van, Turkey

Correspondence to: daksu@yyu.edu.tr

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Abstract: Pomegranate juice (PJ) contains relevant amounts of active biological compounds which alleviate the detrimental effects of chronic heavy metal exposure. This study investigated the protective potential of PJ against lead-induced oxidative stress. A total of forty adult male Sprague Dawley rats were divided into four experimental groups. The animals were fed a standard pellet diet and tap water *ad libitum*. The rats were divided into four groups (n=10 for each group): control, lead asetat (2000 ppm), low-treated PJ- a daily dose of 2.000 ppm lead plus 30µl pomegranate juice (included 1.050 µmol total polyphenols, gallic acid equivalent), and high-treated PJ- a daily dose of 2.000 ppm lead plus 60µl pomegranate juice (included 2.100 µmol total polyphenols, gallic acid equivalent). The treatments were delivered for 5 weeks. After the treatment period, the tissues samples (kidney, liver, heart and testis**)** were collected. Tissue lead (Pb) and mineral amounts (copper, zinc, and iron), tissues lipid peroxidation level and antioxidant status, and tissues histopathological changes were determined. The results showed that the highest rate lead loading was in the kidney and the testis. Pomegranate juice was decreased the lead levels of soft tissues examined; increased Zn amounts in tissues of which the lead accumulation was higher (kidney and the testis); decreased the copper, zinc and the iron levels of the liver and heart tissues, without creating a weakness in antioxidant capacity of these tissues, restricted the oxidative stress by decreasing lipid peroxidation, improved both of the activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalaz (CAT), and the level of glutathione (GSH) in all the tissues examined in lead-treated groups. As histopathological findings, the cellular damage induced by lead in the tissues of the kidney, liver and the heart were observed to have been partially prevented by PJ treatment. The protective effect of PJ was more pronounced in the testis compared to those others.

Key words: Lead; Phenolic compounds; Oxidative stress.

Introduction

Chronic exposure to lead results in its accumulation in vital organs, with serious pathological effects apparent in these organs after a short time of intestinal absorption (1,2). Pathological damages can be occurs in vital organs depending on duration and severity of lead exposure. Lead exposure causes renal dysfunction, liver cirrhosis, damage to the cardiovasculer system and anemia (3). It can also cause some pathological damages in the ovaries and testes, causes by poor reproductive performance (4). Elimination of lead from tissues is slowly. Prolonged exposure to a low level of lead can hereby cause to detrimental effects and disruptions occurs in membrane-related proccess such as the activities of membrane enzymes, endo- and exo-cytosis, transport of solutes across the bilayer and signal transduction processes (5,6). Lead accumulation in tissues causes oxidative stress by inducing the generation of reactive oxygen species (ROS), thereby reducing the anti-oxidant defence system of cells (1). Heavy metals can impair the metabolism of essential minerals (7). Minerals are defined as metal, and they can cause cellular damage and toxicity by interacting with biological macromolecules in the presence of heavy metals such as lead (8,9). From the other side, the cells need trace minerals such as copper (Cu), zinc (Zn) and iron (Fe) to perform their normal biological functions. Therefore, detection and prevention of lead toxicity is a major international public health priority. The current therapeutic strategy for heavy metal toxicity is usually chelating agents. These agents are generally non-specific with respect to metals, and they have many side effects. The lead chelate complex can remain in tissue, and re-distribute to other tissues over time (10). In recent years, studies have begun to focus on the use of anti-oxidants as protectants against the harmful effects of lead (11-13). The pomegranate (Punica granatum, Punicaceae) is consumed fresh or can be processed into juice, jams, or a type of wine. Pomegranate juice contains in a satisfactory amounts of ascorbic acid, and the minerals include Fe, Ca, Mg, Se, Zn, as well as considerable amounts of phenolic compounds (14,15). Pomegranate juice has higher antioxidant capacity than other polyphenol-rich beverages (16). The antioxidant capacity of pomegranate juice in terms of free radical scavenging and iron reduction capacity has been shown to be three times higher than that of red wine and green tea (17). Com-

mercial pomegranate juice is obtained by pressing the whole pomegranate fruit and its peels. Pomegranate juice has considerable antioxidant potential with a high amount of phenolic compounds in comparison to commonly consumed fruit juices, such as grape, cranberry, grapefruit, or orange juice (18,19). Phenolic compounds are natural substances found in plants, fruits, vegetables and plant-derived consumables. These compunds show their mainly antioxidant effects by different mechanisms like inducing expression of protective genes aganist oxidative stress, regulation of reactive oxygen species by interacting with oxidative pathways and scavenging metal ions as pathogenic free radicals (20). Guo et al. (21) reported that 250 mL of pomegranate pulp juice (PPJ) daily for four weeks given to healthy elderly persons increased the plasma antioxidant capacity from 1.33 mmol to 1.46 mmol, while the other's consuming apple juice experienced no significant increase in antioxidant capacity. In addition, plasma carbonyl content (biomarker for oxidant/antioxidant barrier impairment in various inflammatory diseases) significantly decreased in the persons consuming the PPJ compared to the person consuming apple juice. Plasma vitamin E, ascorbic acid, and reduced glutathione values did not differ significantly between the groups, leading researchers to conclude that pomegranate phenolics may be responsible for the observed results. The molecular interactions of phenolic compounds with biological systems continue to be mostly speculative. The free-radical scavenging capability of polyphenols has been primarily tested with *in vitro* studies. However, phenolic compounds are structurally altered *in vivo.* As far as we know, inadequate *in vivo* studies have examined the lead binding activity of PJ, its interaction with minerals (copper, zinc and iron) and the protective effect of PJ against lead-induced oxidative damage. Therefore, the aim of the present study was to evaluate the lead binding activity of PJ, its interactions with the minerals (coper, zinc and iron), and the protective effect on the liver, kidney, heart and the testis of rats exposed to lead.

Materials and Methods

Chemicals

All chemicals were obtained from Sigma Chemical Inc. (St. Louis, MO, USA) and Merck Chemical Inc. (Darmstadt, Germany).

Pomegranate Juice (PJ) preparation

Fresh pomegranate fruit (*Punica granatum*) was purchased from a local retailer. Fresh fruits were washed and then squeezed to remove whole pomegranate juice.

Determination of Total Sugars (TS), Ascorbic Acid (AA) and Total Anthocyanins (TAs) of PJ

In order to estimate TS amount, the method described by Ranganna (22) was used, and the amount was expressed as g L^{-1} of PJ. Amount of AA was determined using the method described by Ruck (23) and expressed as mg L^{-1} of PJ. The total anthocyanins were estimated by the pH differential method using two buffer systems: potassium chloride buffer, pH 1.0 (25 mM), and sodium acetate buffer, pH 4.5 (0.4 M) (24). The total anthocyanins content was calculated as follows:

$$TAs = ((A \times MW \times DF \times 100)/MA)$$

where $A = (A510 - A700)\ pH\ 1.0 - (A510 - A700)\ pH4.5$; MW: molecular weight; DF: dilution factor; MA: molar absorptive coefficient of cyanidin-3-glucosid. Results were expressed as cyaniding-3-glucoside mg L^{-1} of PJ.

Determination of Total Phenolic Content (TPC) and Antioxidant Activity (AA) of PJ

TPC was measured using the Folin-Ciocalteu method described by Ough and Amerine (25) using gallic acid as reference. The antioxidant capacity of pomegranate juice was evaluated based on free antioxidant activity radical scavenging capacity by the DPPH radical (expressed as inhibition percentage)(26).

Determination of copper, zinc, manganese and iron amounts of PJ

The fruit juice sample was analysed for minerals (copper, zinc, manganese and iron) using atomic absorption spectrophotometer (AAS, model AAS-200SN Rotalab).

Animals and experimental design

In this experimental study, forty adult male Sprague Dawley rats (300 ±10 gr) were purchased from an Experimental Research Institue. The animals were housed in plastic cages at 12-light/dark cycle, 21 ± 2°C with water and food available ad libitum. Prior to use, the animals were acclimatized under these conditions for 15 days. The rats were divided into four groups (n=10 for each group): 1) control, 2) lead –a daily dose of 2000 ppm lead asetat alone, 3) low-treated PJ- a daily dose of 2.000 ppm lead plus 30µl pomegranate juice (included 1.050 µmol total polyphenols, gallic acid equivalent), and 4) high-treated PJ- a daily dose of 2.000 ppm lead plus 60µl pomegranate juice (included 2.100 µmol total polyphenols, gallic acid equivalent). The experiments were approved by the local ethical committee at Mustafa Kemal University (B.30.2.M.K.Ü.0.00/05). Lead acetate was dissolved in water and delivered by drinking water, and pomegranate juice was applied by oral gavage. The experiment lasted for five weeks. The dose of PJ treatments was used based on results of previus study (27,28). At the end of the treatments, the rats were anesthetized and sacrificed following an overnight fasting. The organs (kidneys, livers, heart and testis) were dissected and washed in cold ice saline (0.9%) for lipid peroxidation, antioxidant enzyme and molecules, lead and micronutrient analysis. For histopathological examination, tissue sections were obtained from the tissues samples and fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 5 µm thicks, and stained with haematoxylin and eosin (HE). The sections were then examined and photographed under an optical microscope (29). For histopathological examination, tissue sections (kidneys, livers, heart and testis) were quickly removed and processed for histopathology analyses. Tissue sections were fixed in 10% neutral buffered formaldehyde for 48 hours and washed under tap water overnight. Following routine tissue preparation procedures, tissue samples were dehydrated through graded series of alcohol and xylene and embedded in paraffin blocks. Paraffin serial sections were cut at a thickness

of 5 and stained with haematoxylin and eosin (HE). The sections were then examined and photographed under an optical microscope (29). The sections examined on the light microscope were scored as no (-), mild (+), moderate (++) and severe (+++) according to the lesion severity (30). Other parts of tissues homogenate was prepared in an ice cold homogenization buffer (0.32 M sucrose, 1 mM EDTA, 10 mM Tris HCl, pH 7.4), and cytosolic samples of tissues homogenate were obtained by centrifuging at 10.000 g for 10 min at +4ºC.

Lipid peroxidation assay

The tissues lipid peroxidation (as malondialdehyde, MDA) levels were determined using the method described by Yoshoiko et al (31), based on the thiobarbituric-acid (TBA) reaction. The optical density was measured at 535 nm by spectrophotometer (Shimadzu UV 1208).

Lead, copper, zinc and iron estimation in tissues

Tissues were digested with concentrated nitric acid-hydrogen peroxide (2:3) using a micrawave digestion system (speedwave MWS-2 Berghof products + Instruments Harresstr.1. 72800 Enien Germany) (32). The samples were brought to a constant volume and the tissue mineral contents were measured using an inductively couple plasma spectrophotometer (Perkin-Elmer, optima 2100 DV, ICP/OES, Shelton, CT 06484-4794, USA) (33).

Antioxidant enzyme and molecules

The method described by Sedlak and Lindsay (34) was used in order to determine the glutathione (GSH) concentrations of tissues examined. All of the non-protein sulfhydryl groups of cells are in the form of reduced GSH. The chromogen 5,5'-Dithiobis 2-nitrobenzoic acid (DTNB) is a disulfide compound that is readily reduced by sulfhydryl compounds to an intensely yellow compound. The absorbance of the reduced chromogen was measured at 412 nm. Catalase activity in the tissue homogenate was assayed by the decrease in absorbance of hydrogen peroxide at 240 nm as per the method of Aebi (35). Superoxide dismutase activity in the tissue homogenate was assayed spectrophotometrically as described by Sun et al (36). This method is based on inhibition of nitroblue tetrazolium (NBT) reduction by superoxide anions. The resulting reduction of NBT was measured at 560 nm by spectrophotometry. We determined all the tissues' protein levels with the Bradford reagent using a spectrophotometer at 595 nm.

Statistical analysis

The appropriate Sum of Squares Method was selected for ANOVA (37). The data were analyzed by the one-factor ANOVA using the general linear models procedure of SAS (38). Software for the main effect of treatments. The differences between the means were determined by the Duncan's multiple range test at a significance level of $P<0.05$.

Table 1. Estimated chemical paramateres of pomegranate juice.

Total sugars, g L^{-1}	189.41
Ascorbic acid, mg L^{-1}	124.76
Total anthocyanins, mg L^{-1}	88.45
Total phenolics, mg L^{-1} gallic acid equivalent	6645
Total antioxidant activity, %	28.78
Copper, mg L^{-1}	0.28
Zinc, mg L^{-1}	0.39
Manganese, mg L^{-1}	1.96
Iron, mg L^{-1}	2.38

Results

Total Sugars (TS), Total Anthocyanins (TAs), Ascorbic Acid (A), Total Phenolics (TPs), Antioxidant Activity (AA) and mineral (Cu, Zn, Mn, and Fe) concentrations of pomegranate Juice

TS, TAs, A, TPs, AA, and mineral concentrations of pomegranate juice have been presented in Table 1. The results were in accordance with previous reports on other pomegranate cultivars (39,40). The pomegranate juice appears to be a good source of nutrients and variation in the mineral composition could originate from the pomegranate cultivar as well as agro-climatic conditions, handling practices and manufacturing conditions.

Histopathological examination of the tissues

The histopathological changes scores of tissues and examination have been displayed in Table 2-5, and Figure 1-4, respectively. Histopathological examination revealed normal histology of kidney in control group (Figure 1A). Lead administration produced severe hyperemia and infiltration of mononuclear cells in glomerular capillaries and veins in intertubular, dilatation of

Table 2. Histopathological changes scored in kidney tissue.

	Groups			
	Control	Lead	Low-treated PJ	High-treated PJ
Tubul epithelia degeneration	-	+++	+++	+
Tubul epithelia necrosis	-	+++	++	-
Mononuclear cell infiltration	-	+++	++	+
Hyperemia and hemorrhage	-	+++	++	+
Tubul dilatation	-	+++	+++	++

+++: severe; ++:middle; + :little; -: no.

Table 3. Histopathological changes scored in liver tissue.

	Groups			
	Control	Lead	Low-treated PJ	High-treated PJ
Hydropatic degeneration	-	+++	++	+
Hepatocyte necrosis	-	+++	++	-
Mononuclear cell infiltration	-	++	++	-
Hyperemia and hemorrhage	-	+++	+	+

+++: severe; ++:middle; + :little; -: no.

Table 4. Histopathological changes scored in heart tissue.

	Groups			
	Control	**Lead**	**Low-treated PJ**	**High-treated PJ**
Hyaline degeneration	-	+++	++	++
Zenker's degeneration	-	+++	++	-
Mononuclear cell infiltration	-	+++	++	+
Hyperemia and hemorrhage	-	++	+	-

+++: severe; ++:middle; + :little; -: no.

Table 5. Histopathological changes scored in testis tissue.

	Groups			
	Control	**Lead**	**Low-treated PJ**	**High-treated PJ**
Atrophy in seminiferous tubules	-	+++	++	-
Intertubular edema	-	++	+++	+
Slimming on the tubul wall	-	+++	++	-
Active spermium	+++	-	+	++

+++: severe; ++:middle; + :little; -: no.

tubules, degeneration and necrosis of tubuler epithelium (Figure 1-B). In low-treated PJ group, hyperemia in tubular glomerular capillary and intertubuler range, dilatation in the tubule lumen, degeneration in the tubuler epithelium were observed in kidney tissues (Figure 1-C). In high-treated PJ group, dilatation in tubules and very few degeneration in tubular epithelium vessels with nearly normal histological structures were determined (Figure 1-D). For liver tissue, normal histological structures were observed in control group (Figure 2-A). In the group receiving lead alone, diffuse hydropic degeneration, necrosis, hyperemia in sinusoidal and acinar veins were detected (Figure 2-B) whilst degeneration in acinar region, few necrotic hepatocytes, sinusoidal dilatation and hyperemia were determined in low-treated PJ (Figure 2-C). In high-treated PJ group, a few number of degenerated hepatocytes in the acinar region, mild hyperemia (Figure 2-D) were observed in liver tissues. Also, histopathological examination of the heart sections of control group showed normal cardiac myocytes (Figure 3-A). In the group receiving lead alone, the common hyperemia and perivascular mononuclear cell infiltration, hyaline degeneration and Zencker's necrosis in the myocardium were detected (Figure 3-B). In low-treated PJ group, mononuclear cell infiltration, hyaline degeneration and a small number of Zencker's necrosis were determined (Figure 3-C). In high-treated PJ group, a few number of degenerated cells and interstitial hyperemia were observed (Figure 3-D). For testis tissue, normal histological structure were observed in control group (Figure 4-A). Lead administration produced atrophy of seminiferus tubular, thinning of the tubule wall due to

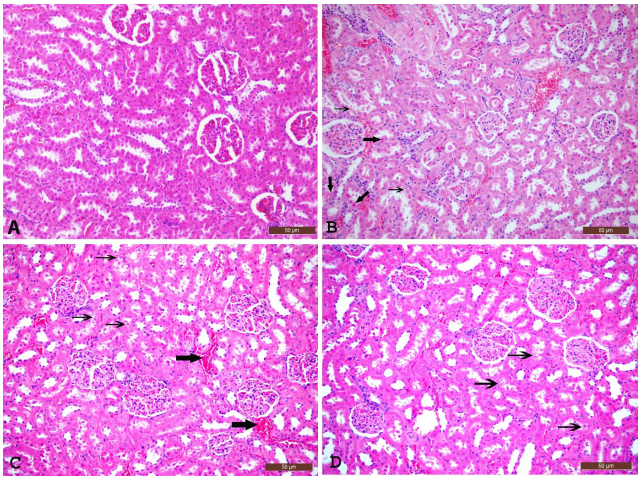

Figure 1. A. normal histological appearance of kidney tissue of control group, Bar: 50µm; B: kidney tissue of the rat received 2000 ppm lead alone; severe hyperemia and infiltration of mononuclear cells in glomerular capillaries and veins in intertubular, dilatation of tubules, degeneration (thin arrows) and necrosis of tubuler epithelium (thick arrows), Bar: 50µm; C: kidney tissue of the rat received 2000 ppm lead plus 30µl pomegranate juice (low-treatment); hyperemia (thick arrows), hyperemia in tubular glomerular capillary and intertubuler range, dilatation in the tubule lumen, degeneration in the tubuler epithelium (thin arrows), Bar: 50µm. D: kidney tissue of the rat received 2000 ppm lead plus 60µl pomegranate juice (high-treatment); dilatation in tubules and very few degeneration in tubular epithelium (arrows) Bar: 50µm.

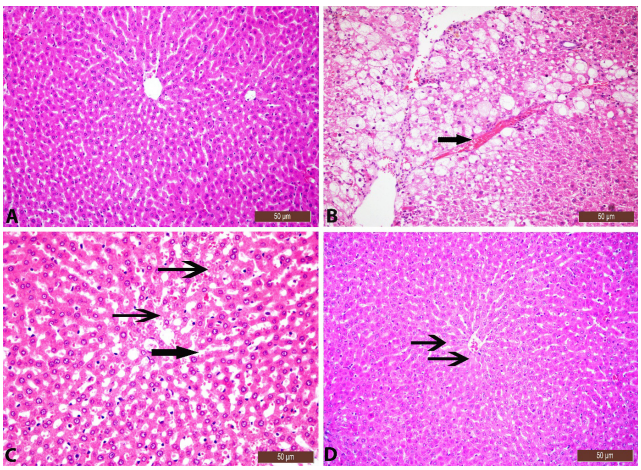

Figure 2. A. normal histological appearance of liver tissue of control group, Bar: 50µm; B: liver tissue of the rat received 2000 ppm lead alone; diffuse hydropic degeneration, necrosis, sinusoidal and sentral veins hyperemia (arrows), Bar: 50µm; C: liver tissue of the rat received 2000 ppm lead plus 30µl pomegranate juice (low-treatment); degeneration (thin arrow) in central region and few necrotic hepatocytes, sinusoidal dilatation (thick arrow) and hyperemia, Bar: 50µm; D: liver tissue of the rat received 2000 ppm lead plus 60µl pomegranate juice (high-treatment); a few number of sentral degenerated hepatocytes (arrows), mild central hyperemia, Bar: 50µm.

reduction in germ cells, the absence of spermetazoa in some tubule lumen. A small number of spermetazoa in some tubule lumen were detected in the group receiving lead alone (Figure 4-B). In low-treated PJ group, degeneration of germ cell and intertubular edema, a small number of spermetazoa in tubule lumen were observed (Figure 4-C). In high-treated PJ group, mild edema in intertubuler range was determined whilst many sperme-

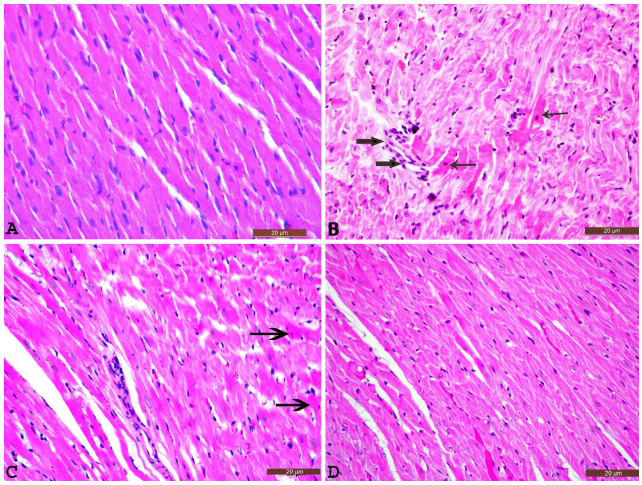

Figure 3. A. normal histological appearance of heart tissue of control group, Bar: 20µm; B: heart tissue of the rat received 2000 ppm lead alone; common heart hyperemia and perivascular mononuclear cell infiltration (Thick arrow), hyaline degeneration and necrosis (thin arrow), Bar: 20µm; C: heart tissue of the rat received 2000 ppm lead plus 30µl pomegranate juice (low-treatment); mononuclear cell infiltration, hyaline degeneration and in a small number of necrosis (arrows), Bar: 20µm; D: heart tissue of the rat received 2000 ppm lead plus 60µl pomegranate juice (high-treatment); a few number of degenerated cells (arrows) and vascular hyperemia. Bar: 20µm.

tazoa were also found (Figure 4-D).

Tissues' lead (Pb) and mineral levels

Table 6 shows the lead, copper, zinc and the iron levels of the kidney, liver, heart and the testis tissues. The lead levels of the tissues in the group that received a daily dose of 2.000 ppm lead (as lead acetate) were highest compared to those others (P<0.001). In the present study, the highest lead accumulation was observed in the kidney. The rate of lead accumulation was 13.7 times higher in the kidneys of the lead-exposed rats compared to the controls. It was 8.6 times higher in the testis and 2.5 times higher in the heart and liver of the lead-exposed rats compared to the controls. Experimental application of lead increased the zinc accumulation in the kidney; it also increased the iron accumulation in the liver, heart and the testis tissues (P<0.001). Contrarily, the lead application decreased the copper and zinc levels in the testis (P<0.001). Furthermore, the copper and iron levels of the kidney, and the copper and zinc levels of the liver and the heart did not change by the experimental application of lead. The levels of both phenolic compounds decreased the lead amount of examined tissues compared to the group that received lead alone (P<0.001). Additionaly, both phenolic compounds levels further increased the zinc amount of kidney tissue compared to the the group that received lead alone. Phenolic compunds at 60 µl level significantly decreased the copper, zinc and iron amounts of liver tissue, even below the value of the control group (P<0.001). Both phenolic compounds levels decreased the copper and zinc amounts of heart tissue compared to those of the controls and the group that received lead alone (P<0.001). On the other hand, phenolic compunds at 30 µl level did not affect the iron accumulation in heart tissue, while phenolic compunds at 60 µl level significantly decreased the iron amount, even down to

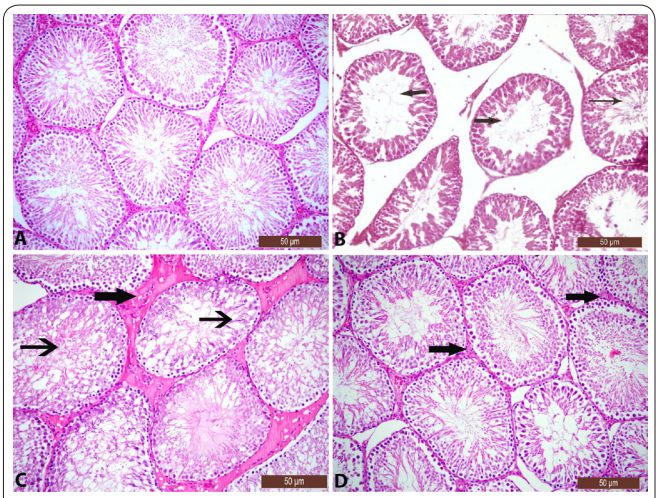

Figure 4. A. normal histological appearance of tubules seminiferi of rat testis tissue of control group, Bar: 50µm; B: testis tissue of the rat received 2000 ppm lead alone; atrophy of seminiferus tubular, thinning of the tubule wall due to reduction in germ cells that make up tubuler wall, the absence of spermetazoa in some tubule lumen (thick arrow), in a small number of spermetazoa in some tubule lumen (thin arrow), Bar: 50µm; C: testis tissue of the rat received 2000 ppm lead plus 30µl pomegranate juice (low-treatment); degeneration of germ cell and intertubular edema,(thick arrow) fewer making spermatozoa (thin arrow), Bar: 50µm; D: testis tissue of the rat received 2000 ppm lead plus 60µl pomegranate juice (high-treatment); mild edema in intertubuler range (arrows), Bar: 50µm.

the iron level of the control group (P<0.01). In the testis, the reduced levels of copper in the group receiving lead alone were increased to the control level in both groups that received PJ. The inreased levels of iron in the group receiving lead alone were also decreased to that of control level in both of groups that received PJ (P<0.001). Furthermore, the amount of zinc was increased in both PJ levels, even higher than that of the controls (P<0.001).

Lipid peroxide levels and antioxidant parameters

Lipid peroxidation, determined by MDA formation, was increased in all examined tissues of the rats exposed to lead while PJ administration significantly decresed the level of lipid peroxidation in all tissues. When the group receiving lead alone was compared with the controls, it was observed that the GSH level was significantly decreased in all tissues while SOD and CAT activities were significantly increased. The GSH levels in both of the groups receiving PJ were significantly lower than that of the controls, although its level was significantly higher than the group receivinglead alone (P<0.001). In particular, the GSH levels of heart muscle in the group receiving 60 µl PJ was very close to that of the controls. When examining the CAT and the SOD activities of the groups receiving both levels of pomegranate juice, it was observed that CAT activities in all tissues were lower than the group receiving lead alone, while it was higher than the controls' level. The SOD activities in the heart and the testis tissues of the group receiving60 µl PJ were close to that of the control group (P<0.001).

Table 6. Tissue lead (Pb) and mineral amounts of experimental groups.

	Control	Lead	Low-treated PJ	High-treated PJ	
Items, mg/kg	**Kidney**				P
Lead	0.03 ^{d} ±0.002	0.41^{a}±0.034	0.18^{b}±0.016	0.09^{c}±0.001	0.001
Copper	5.11±0.295	5.45±0.505	5.56±0.465	4.52±0.223	0.249
Zinc	9.05^{d}±0.091	11.87^{c}±0.145	16.64^{a}±0.799	15.01^{b}±0.374	0.001
Iron	125.19±3.776	119.18±1.455	123.28±4.918	121.74±4.380	0.732
	Liver				
Lead	0.11^{d}±0.002	0.26^{a}±0.012	0.19^{b}±0.003	0.14^{c}±0.003	0.001
Copper	2.88^{a}±0.091	2.64^{a}±0.138	2.82^{a}±0.153	2.20^{b}±0.035	0.001
Zinc	41.66^{a}±0.844	41.65^{a}±1.260	38.72^{ab}±1.192	37.77^{b}±0.427	0.013
Iron	36.37^{b}±0.631	39.32^{a}±0.717	39.87^{a}±0.536	33.01^{c}±0.490	0.001
	Heart				
Lead	0.03^{d}±0.001	0.08^{a}±0.002	0.06^{b}±0.001	0.04^{c}±0.001	0.001
Copper	0.50^{a}±0.011	0.49^{a}±0.012	0.42^{b}±0.021	0.32^{c}±0.008	0.001
Zinc	35.59^{a}±1.814	38.67^{a}±0.727	31.35^{b}±0.819	30.81^{b}±0.927	0.001
Iron	6.06^{b}±0.339	7.54^{a}±0.274	7.11^{a}±0.187	5.85^{b}±0.210	0.001
	Testis				
Lead	0.17^{d}±0.011	1.47^{a}±0.095	0.57^{b}±0.021	0.41^{c}±0.025	0.001
Copper	3.62^{a}±0.091	3.06^{b}±0.084	3.90^{a}±0.180	3.64^{a}±0.202	0.003
Zinc	53.54^{b}±2.108	47.31^{c}±1.625	64.38^{a}±2.312	63.42^{a}±2.505	0.001
Iron	151.58^{b}±4.65	180.99^{a}±3.14	157.21^{b}±5.211	162.30^{b}±4.648	0.001

$^{abcd:}$ Mean values within the same row labelled different letters are statistically significant (P<0.001).

Table 7. Lipid peroxide levels and antioxidant status of experimental groups.

	Control	Lead	Low-treated PJ	High-treated PJ	
Tissues	**MDA (µmol/mg protein)**				P
Kidney	12.51^{d}±0.28	17.72^{a}±0.33	15.95^{b}±0.14	14.72^{c}±0.18	0.001
Liver	12.01^{d}±0.39	17.15^{a}±0.19	15.28^{b}±0.15	14.42^{c}±0.06	0.001
Heart	22.65^{c}±0.37	44.66^{a}±2.30	29.14^{b}±2.94	25.51^{c}±0.22	0.001
Testis	7.25^{b}±0.43	13.20^{a}±0.99	12.13^{a}±0.79	7.35^{b}±0.41	0.001
	CAT (U/mg protein)				
Kidney	1.34^{c}±0.02	1.90^{a}±0.04	1.59^{b}±0.07	1.48^{b}±0.03	0.001
Liver	0.26^{c}±0.02	1.22^{a}±0.08	0.70^{b}±0.05	0.60^{b}±0.03	0.001
Heart	0.28^{b}±0.01	0.57^{a}±0.07	0.38^{b}±0.08	0.33^{b}±0.03	0.001
Testis	0.11^{b}±0.01	0.33^{a}±0.04	0.14^{b}±0.01	0.10^{b}±0.01	0.001
	SOD (U/mg protein)				
Kidney	0.88^{c}±0.03	1.28^{a}±0.03	1.11^{b}±0.03	1.06^{b}±0.04	0.001
Liver	1.17^{c}±0.05	1.78^{a}±0.07	1.56^{b}±0.08	1.41^{b}±0.08	0.001
Heart	0.22^{c}±0.02	0.34^{a}±0.01	0.27^{b}±0.01	0.25^{bc}±0.01	0.001
Testis	0.76^{b}±0.04	1.01^{a}±0.06	1.01^{a}±0.04	0.87^{ab}±0.04	0.001
	GSH (µmol/mg protein)				
Kidney	0.77^{a}±0.02	0.53^{c}±0.02	0.63^{b}±0.01	0.68^{b}±0.02	0.001
Liver	6.45^{a}±0.27	2.77^{d}±0.05	3.92^{c}±0.21	5.09^{b}±0.28	0.001
Heart	0.63^{a}±0.02	0.37^{c}±0.01	0.53^{b}±0.03	0.58^{ab}±0.03	0.001
Testis	0.35^{a}±0.01	0.24^{c}±0.02	0.28^{b}±0.02	0.31^{b}±0.02	0.001

$^{abcd:}$ Mean values within the same row labelled different letters are statistically significant (P<0.001).

Discussion

To our knowledge, an inadaquate number of studies has been carried out to determine the protective effects of phenolic compounds in pomegranate juice on mammalian tissues against lead accumulation and lead induced oxidative damage although the structure and mode of action of phenolic compounds and pomegranate juice have been discussed. We therefore investigated the ameliorative effects of pomegranate juice against lead accumulation, the lead binding activity, interactions with minerals (copper, zinc and iron) and the protective effect on the liver, kidney, heart and testis of rats exposed to lead.

In the current study, the highest lead accumulation was observed in the kidney. Kidney cells can concen-

trate the lead in intranuclear inclusion bodies which are storage sites that may have a protective effect during excessive lead exposure (42). The histopathological findings in kidney tissue in this study are also supported by this statement. Lead exposure has affected the mineral amounts in tissues examined. According to this; lead exposure increased the zinc loading in the kidney; it increased the Fe loading in the liver, heart and the testis tissues. On the other hand, lead exposure decreased the loading of copper and zinc of the testis whilst it did not affect the amounts of copper and iron of the kidney, and the amounts of copper and zinc of the liver and the heart, respectively. It is known that zinc homeostasis is regulated by the kidney and that a small amount is excreted through bile (42). We also thought that the lead-induced kidney damage observed as pathological results may have resulted in a reduction in the urinary excretion of zinc; thus, Zn deposition may be increased in the kidneys. Another important finding of this study is that lead exposure resulted in the accumulation of iron in the liver, heart and the testis (Table 6). Iron is an essential element, and it is necessary for normal cellular functioning (43). Iron and lead occupy similar niches within the body. Therefore, they compete for similar binding sites, particularly during absorption (44). In doing so, the increased lead absorption in the gastrointestinal system may cause a limitation in iron absorption. When iron is deficient, hepatocytes produce less or no hepcidin (45). Iron loading in the liver and other tissues develops in animals with reduced hepcidin expression (46). In the present study, exposure to lead decreased the testicular copper and zinc amounts. The testes requires a high zinc concentration to maintain their normal physiology. Zinc maintains the redox balance by modulating several Zn-dependent enzyme metallothioneins (MT) (47). Metallothioneins are metal-binding proteins that are cysteine-rich and of low molecular weight. In fact, metallothioneins have a protective effect against toxic metals. Although the specific region for heavy metals-binding of metallothioneins is lower in number, their expressions are increased in the presence of heavy metals such as cadmium, lead and mercury (48). The metal-binding regions of metallothioneins also have specific areas for copper and zinc. The metal binding capacity of metallothioneins is directly related to the metal density in cells (45).

The reduction in the tissues' lead amounts in the rats that received PJ could be attributed to the lead-fixing activity of polyphenolic compounds. As far as we know, there is no previously study expressing these results, but the present findings showed that phenolic compounds in PJ decreased the tissusue lead amounts. Two attachment sites for metal ions have been suggested within the molecular structure of flavanols, which are chemical compounds of polyphenols; the o-diphenolic groups in the 30,40-dihydroxy positions in the B ring and the keto structure 4-keto, 3-hydoxy or 4-keto and 5-hydroxy in the C ring. These functional groups bind to Cu and Fe ions. With a closer look at the molecular structures, the same chemical and molecular features can also be found in other chemical components of phenolic compounds (49). We can therefore speculate that polyphenols in PJ may have the ability of binding the lead as well as copper and iron. Although the binding of metals, such as copper and iron, by phenolic compounds can reduce the bioavailability of these metals, this negative effect can also be advantageous in organisms. Copper and iron participate in Fenton and Heber–Weiss reactions, generating highly reactive hydroxyl radicals, which are associated with many pathological conditions (50). The binding of these metals may decrease the hydroxyl radical-induced cell toxicity. As a remarkable finding, the zinc amount also increased in the kidney and the testis, to which the most lead was loaded when pomagranate juice were applied. This finding has drawn attention onto the protective effects of pomagranate juice in lead exposure once again. High Zn concentration is essential for the normal physiology of the testes and Zn homeostasis is regulated by the kidney (51). A previous study indicated that zinc acts as an anti-oxidant and possibly as a chelator agent in lead toxicity, and PJ is rich in Zn (52). The reason for the zinc loading in the kidney and the testis tissues can be attributed to this mechanism.

As expected, the lead exposure enhanced the generation of ROS and lipid peroxidation, and caused cell damage. Lipid peroxidation is a well-known mechanism of oxidative damage caused by ROS and it has been used a potential marker of oxidative stress. In this study, the increases in lipid peroxidation (indicated by a high MDA level) in the tissues of lead-exposed rats were altered in the animals' antioxidant defence systems, including decreased GSH levels in all tissues examined and increased SOD and CAT activity (Table 7). The increase in SOD and CAT activities in all the tissues of the rats exposed to lead alone could be a compensation mechanism to counteract the decreasing level of GSH. Glutathione (GSH) contains reactive sulfhydryl groups (-SH), which play an important role in protecting the cell membrane lipids from ROS attack. However, GSH is rapidly oxidized by oxidants (53). Lead causes an elongation in fatty acids by increasing the number of double bonds, thereby increasing the lipid peroxidation in the cell membranes. Additionally, the affinity of lead for sulfhydryl groups (-SH) adversely affects the integrity of cell membranes (54). Peroxidation of the phospholipid structure in cells leads to deterioration of cell membrane integrity and ultimately to cell death (5). Thus, the histopathological findings such as cell damage in the glomerulus, liver hepatocytes, heart muscle cells and semineferous tubules observed in the group receiving lead alone indicated the cell damage. These histopathological findings were supported by previous studies (55,56). Superoxide dismutase and CAT are important enzymatic antioxidants involved in protecting the cells against the detrimental effects of free radicals. SOD is a well-known major antioxidant enzyme containing copper and zinc (57). In the current study, increasing the supplementation level of PJ decreased the copper and zinc amounts, even to a level lower than those of controls, but this reduction did not create a weakness in SOD activity of the liver and the heart (Table 6 and 7). CAT is a heme-containing antioxidant enzyme, which acts sequentially to SOD in the conversion of hydrogen peroxide to water. Fe+3 protoporphyrin is a major component of CAT, and CAT activity is related to the copper level (58). Copper is necessary for adequate utilization of iron, which is an important component of CAT. Similarly, increasing the supplementation level of PJ decreased the copper and

iron levels of the liver and the heart, but this reduction did not create a weakness in CAT activity in these tissues (Table 6 and 7). According to these findings, reduction of the amounts of copper-, zinc- and iron caused by phenolic compounds in the PJ did not create any negative impact on either of the antioxidant activities SOD and CAT in the liver and heart tissues. The chelation of these micronutrients may be beneficial in living organisms by inhibiting the oxidative damage in these tissues. The close link between these minerals (copper and iron) and Fenton and Heber–Weiss reactions that are generating highly reactive hydroxyl radicals, which are associated with many pathological conditions is well known (50). In this study, PJ could alleviate the oxidative damage by decreasing the lipid peroxidation (indicated by low MDA level), improving the activities of antioxidant enzyme (CAT and SOD) and GSH level (Table 7). This contrubution could be attributed to the presence of ascorbic acid, total anthocyanins, total phenolics and total antioxidant activity in PJ (Table 1). In agreement with the present study, Wie et al., (59) found that treatment with EPP (the extracts of pomegranate peels, 150mg/kg body weight) and EPS (the extracts of pomegranate seeds, 100mg/kg body weight) markedly decreased the hepatic MDA levels and efficiently increased the hepatic SOD and GSH-Px activities compared with that of the carbon tetrachloride (1mL/kg body weight) injected groups in rats. Treatment with EPP and EPS obviously alleviated the collagen deposition and liver injury. In another study conducted by Reckziegel et al., (60), the antioxidant protection of gallic acid (GA) against toxicity induced by Pb in rats were investigated. It was observed that an increase in Pb levels in blood, liver and kidney ($P<0.001$) in Pb-exposed rats in relation to control group. This affect was not reverted by GA treatment. SOD activity was decreased in liver and kidney of Pb-exposed rats in relation to control group, and the events were partially reverted in liver and completely in kidney by GA. In liver CAT activity decreased in Pb-exposed rats compared with control group, and this affect reversed by GA treatment ($P<0.05$). No changes were observed in kidney CAT activity (60). These compounds have protective effects against the generation of high reactive species such as hydroxyl radicals ($OH^{\cdot}$), hydrogen peroxide (H_2O_2) and superoxide anions ($O_2^{\cdot-}$) (16). On the other side, the polyphenolic compounds in PJ did not exactly repair the tissue damage compared to the normal cell structure, but showed an ameliorative effect against lead-induced pathological damages on the rat tissues examined, especially in the testes. This effect was especailly pronounced in the group that received 2100µmol polyphenols (high treated group) by pomagranate juice, such that lead-induced pathological damages were mild or of low intensity in this group compared to those of others. Oyagbemi et al. (61) observed the histopathological changes like degeneration and coagulation necrosis in tubul epithelium, mononuclear cell infiltration in inter tubular spaces, and severe hyperemia and hemorrhage in the kidneys of Pb-exposed rats (0.25, 0.50, and 1.0 mg/mL of lead acetate). They reported an increase in the severity of lesions with increasing Pb-doses. Similarly, Abdou and Hassan (3) reported hepatic histopathological changes like degeneration and necrosis in hepatocytes, dilatation in portal veins, hyperemia and haemorrhage, mononuclear cell infiltration in portal region in the liver of cadmium-exposed rats. Kolawole et al.(4) observed a significant reduction in the number of sperm and motility, and histopathological changes like significant thinning in the wall of the testis tubules, deterioration of the tubulosic structure in the rats that received 2.25mg/kg body weight of lead acetate (4). Muhammed (62) reported that the phenolic compound extract of green tea protected kidney tissues against toxicity effect of cadmium sulphate by improving the histopathological alterations and normalizing the kidney biochemical parameters. A group which orally gavage with phenolic compound extract of green tea (at a dose 400mg/kg of body weight) showed high cellularity glomeruli and normal renal convoluted tubules which lining with normal endothelial cells (62). In a new study (63) where avacado fruit extract (AFE) and avacado seed extract (ASE) were used polyphenols-rich ingredients in diethylnitrosamine/2-acetylaminoflurine (DEN/2AAF)-exposed rats, it was observed that the DEN/2AAF-administration created histopathological changes in both of kidney and heart like mononuclear leucocytic aggregation and perivascular oedema, severe vacuolar degenerative changes of the epithelial lining of renal tubules, brown pigments accumulation and few karyomegalic nuclei, perivascular oedema, intramuscular oedema, degeneration of certain cardiomyocytes and intramuscular haemorrhage, respectively (63). The treatment of AFE and ASE showed congestion in the blood vessels with nearly normal histological structure in kidney, and no histopathological changes in heart (63). The histopathological findings of the previously mentioned studies are consistent with our findings.

It was concluded that the ameliorative effect of PJ on metal detoxification was carried out with ascorbic acid, minerals including Fe, Ca, Mg, Se, Zn, as well as considerable amounts of phenolic compounds together. As a result, regular consumption of pomagranate juice could be useful in protecting from chronic lead exposure, which is an important public health problem.

Author agreement

The authors have no conflicts of interest to identify. All of the authors have reviewed and approved this manuscript prior to submission. This manuscript describes the original work of the authors and has not been previously considered for publication.

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