

Original article**The protective role of polyphenols on blood cells in rats exposed to lead****Rolul protectiv al polifenolilor asupra celulelor sanguine la șobolanii expuși la plumb**Devrim Sarıpınar Aksu^{1*}, Mustafa Didin², Fatma Kayıkci³*1. Department of Physiology, Faculty of Veterinary Medicine, Mustafa Kemal University, Hatay-Turkey**2. Department of Food Hygiene, Faculty of Agriculture, Mustafa Kemal University, Hatay-Turkey**3. Department of Histology and Embryology, Faculty of Veterinary Medicine, Selcuk University, Konya-Turkey***Abstract**

The present study investigated the protective potential of the polyphenols in pomegranate juice against the detrimental effects of lead exposure on the hematological system and antioxidant parameters of rat red blood cells. Forty adult male Sprague Dawley rats weighing about 300 g were allocated randomly to four groups: a control group that received normal food and water; a positive control group that received a daily dose of 2000 ppm lead (as lead acetate) in their drinking water for 5 weeks; a low treatment group that received a daily dose of 2000 ppm lead together with 30μl pomegranate juice (PJ; equivalent to 1050 μmol total polyphenols) by gavage for 5 weeks; and a high treatment group that received 2000 ppm lead and 60μl PJ (equivalent to 2100 μmol total polyphenols) daily for 5 weeks. The plasma lead level was significantly ($p < 0.001$) decreased as the plasma copper and zinc levels were significantly increased ($p < 0.001$) in rats received PJ. Significant increases ($p < 0.001$) in red blood cell (RBC) and white blood cell (WBC) counts, and haemoglobin (Hb) and packet cell volume (PCV) were found in both PJ groups compared to the rats exposed to lead alone. Rats that received PJ in addition to lead showed erythrocyte glutathione and plasma ceruloplasmin levels and erythrocyte superoxide dismutase and catalase activities that were almost close to the control values. These observations indicated that treatment of rats with polyphenols might have produced amelioration in hematological system of rats exposed to lead, protecting the level of copper, zinc and antioxidants by against lead induced damage in blood cells.

Keywords: Lead, polyphenols, pomegranate juice, oxidative stress, blood cell, rat

Rezumat

Studiul de față a investigat potențialul de protecție al polifenolilor din sucul de rodie împotriva efectelor negative ale expunerii la plumb în sistemul hematologic și parametrii antioxidanți ai globulelor roșii din sânge șobolan. Patruzeci de șobolani Sprague Dawley adulți de sex masculin, cu greutate de aproximativ 300 g au fost

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distribuiți în mod aleatoriu în patru grupuri: un grup de control care a primit hrană și apă normale, un grup de control pozitiv, care a primit o doză zilnică de plumb 2000 ppm (sub formă de acetat de plumb), în apa lor de băut pentru 5 săptămâni, un grup de tratament cu doză mică, care au primit o doză de plumb zilnică de 2000 ppm, împreună cu suc de rodie 30μl (echivalentul a 1050 micromoli de polifenoli) timp de 5 săptămâni și un grup de tratament cu doză mare, care a primit 2000 ppm plumb și 60μl suc de rodie (echivalentul a 2100 micromoli de polifenoli) pe zi, timp de 5 săptămâni. Nivelul de plumb plasmatic a scăzut semnificativ ($p < 0.001$), iar concentrațiile plasmatiche de cupru și de zinc au crescut semnificativ ($p < 0.001$) la șobolanii care au primit suc de rodie. Creșteri semnificative ($p < 0.001$) ale numărului de hematii și leucocite, precum și a hemoglobinei (Hb) și hematocritului s-au obținut la ambele grupuri tratate cu suc de rodie, comparativ cu șobolanii expuși doar la plumb. La șobolanii care au primit pe lângă doza de plumb și suc de rodie, nivelul glutathionului, activitatea superoxid dismutazei și catalazei în hematii și nivelurile plasmatiche ale ceruloplasminei au fost aproape de valorile de control. Aceste observații au indicat faptul că tratamentul cu polifenoli la șobolanii expuși la plumb, ar putea produce ameliorarea parametrilor sistemului hematologic, protejarea nivelului de cupru, zinc și antioxidanți împotriva daunelor induse de plumb în elementele figurate sanguine.

Cuvinte cheie: Plumb, polifenoli, suc de rodie, stres oxidativ, elemente figurate sanguine, șobolan

Introduction

Oxidation is an important metabolic function that provides energy for cellular activities. However, excessive intracellular oxidation leads to the production of free radicals in the form of reactive oxygen species (ROS) (1). These ROS, which include superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot OH$), are implicated in oxidative damage to various cellular macromolecules (2). Free radicals attack unsaturated fatty acids of biomembranes, resulting in lipid peroxidation, and they also destroy proteins and DNA, which in turn causes a series of deteriorative changes in biological systems leading to cellular inactivation (3).

Lead poisoning is a major human health problem. Lead, which performs no useful physiological function in the body, is taken up into the body from the air, water, soil, and food sources and causes many physiological, biochemical, and behavioral disorders (4). Major sources of lead exposure include lead-based paints, materials used in these paints, glazed porcelain and ceramic materials, lead-acid batteries used in automobiles, lead-soldered canned packages, water contaminated with lead, vegetables and fruits from land contaminated with lead, tobacco products, fish from lead-contaminated environments, white and red meat, and milk and milk products (5). Heavy traffic is

also an important source of lead (6). Because lead is slowly excreted from the body, prolonged exposure to low amounts of lead can cause a number of toxic effects due to accumulation in the body (7). In vitro and in vivo studies have shown that lead exposure promotes the production of reactive oxygen species (ROS). Elevated levels of reactive oxygen species in turn cause lipid peroxidation, DNA damage, and the depletion of cell antioxidant defense systems (8). One of the main targets of lead toxicity is the hematological system. Recent studies have suggested that lead induces oxidative stress in red blood cells (4,9). Red blood cells have a high affinity for lead (10). There are number of factors which contribute to its sensitivity towards lead that include: a) RBC are exposed to high concentration of oxygen; b) hemoglobin can be easily auto oxidized; (c) RBC membrane components are vulnerable to lipid peroxidation, and d) RBC's have limited capacity to repair their damaged components (11). The enzyme δ -Aminolevulinic acid dehydratase (ALAD) is a sulfhydryl-containing protein that catalyzes the asymmetric condensation of two δ -Aminolevulinic acid (ALA) molecules to yield porphobilinogen, a HEM precursor. This enzyme is highly sensitive to the toxic effects of lead (12). High levels of lead may disrupt the HEM biosynthesis, by preventing substrate binding to sulfhydryl groups on ALAD (13). Lead inhibits aminolevulinic acid dehydratase (ALAD), one of the

enzymes that catalyzes the synthesis of δ -aminolevulinic acid (ALA) from phorphobilinogen, and this leads to ALA accumulation in erythrocytes. The resulting accumulation of ALA then may become an important source of free radicals. Free radicals would then cause lipid peroxidation by attacking the unsaturated fatty acids in cell membranes (14). The activity of ferrochelatase, an enzyme that binds Fe^{2+} to protoporphyrin in the last step of the synthesis of heme (HEM), is also blocked by lead. A decrease in ALAD and ferrochelatase activities results in anemia, due to inhibition of HEM synthesis. On the other hand, increased levels of ALA promote ROS production and oxidative stress (15).

Several reports have also suggested that exposure to lead suppresses the humoral immune response, causes functional impairment of lymphocytes, and increases the production of cytokines. Thus, lead exposure reduces humoral and cell mediated immunity and diminishes host resistance, and T cell function is a target for lead (16). Other studies on lead-exposed animals and human workers have reported a number of alterations in antioxidant enzyme activities, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and changes in the concentrations of some key antioxidant molecules, such as glutathione (9,16). Polyphenols are natural antioxidant substances found in plants, fruits, and vegetables, and in plant derived consumables such as olive oil, red wine, and tea. Flavonoids comprise the largest group of polyphenols and are mainly divided into anthocyanins (the glycosylated derivative of anthocyanidin, present in colorful flowers and fruits and anthoxanthins) and colorless compounds further divided into several categories including flavones, isoflavones, flavanols, flavans, and flavonols (17). The importance of polyphenolic flavonoids lies in their antioxidant properties that can enhance cell resistance to oxidative stress. However, these properties go well beyond simple scavenging activity and are of particular interest in pathologies in which

oxidative stress plays an important role. Edible plants provide the human diet with more than 8000 different polyphenols. The pomegranate has been used in the folk medicine of many cultures and pomegranate juice (PJ) is rich in antioxidants of the polyphenolic flavonoid class, including organic acids, tannins, and anthocyanins. Both organic acids and phenolic compounds are important for their potential health benefits. The soluble polyphenolic contents of pomegranate juice include anthocyanins, gallic, ellagic, caffeic, ferulic, and coumaric acids and some flavonoids such as catechin, quercetin, and phloridzin. These flavonoids are powerful antioxidants, whose activities correlate with their chemical structures (18,19).

In recent years, studies have begun to focus on the use of antioxidants as protectants against the harmful effects of lead, as a corollary to traditional treatments that involve the use of chelate-forming agents or removal of accumulated lead from tissues (4, 9, 20). In considering that the high interest of lead to the erythrocytes and erythrocytes are responsible for the distribution of lead in the body, it must firstly thought that hematological system should be protected to minimize detrimental effects of lead on organism.

The free-radical scavenging capability of polyphenols, such as those found in PJ, has been primarily tested in *in vitro* studies; however, no *in vivo* data are yet available describing the metal binding activity and protective effects of PJ phenolic compounds on the hematological system, as far as we are aware. Therefore, the aim of the present study was to evaluate the metal binding activity and protective effect of PJ polyphenols on the hematological properties of blood cells from rats following lead exposure.

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 and Determination of Total Phenolic Content

Fresh pomegranate fruit (*Punica granatum*) was purchased from a local retailer. Fresh fruits were washed and then squeezed to remove the extract. Total phenolic content in this pomegranate juice extract was determined as described by Ough and Amerine (21) and expressed as gallic acid equivalents ($\mu\text{mol/L}$). One milliliter of extract was mixed with 60 ml of distilled water. This solution was mixed again following the addition of 5 ml Folin-Ciocalteu reagent. After 30 seconds, 15 ml of 20% sodium carbonate solution was added and mixed. The extract was filtered through Whatman No.41 filter paper and the mixtures were stored at 20° C for 2 hours. The absorbance was then measured at 765 nm using a spectrophotometer.

Animals and Treatments

Forty adult male Sprague Dawley rats, each weighing about 300 g, were obtained from Firat University, Experimental Research Unit, Faculty of Medicine, Elazığ/Turkey. The animals were fed a commercial diet (Elazığ Feed Factory, Elazığ, Turkey) and tap water *ad libitum* through the experiment. The rats were housed in individual stainless-steel cages (300x240x140mm), each containing two animals. All animals were kept under standard laboratory conditions (light period 07.00 a.m. to 8.00 p.m., 12 h, at 21±2 °C, relative humidity 55%). Prior to use, the animals were acclimatized under these conditions for 15 days. All experiments described in this study were approved by the Ethical Committee of the Mustafa Kemal University (B.30.2.M.K.Ü.0.00/05).

Rats were randomly divided into four groups, each containing ten rats; a control group that received normal food and water; a positive control group that received a daily dose of 2000 ppm lead (as lead acetate) in their drinking water for 5 weeks; a low treatment group that received a daily dose of 2000 ppm lead together with 30 μl pomegranate juice (PJ; equivalent to 1050 μmol total polyphenols) by gavage for 5 weeks; and a

high treatment group that received 2000 ppm lead and 60 μl PJ (equivalent to 2100 μmol total polyphenols) daily for 5 weeks.

At the end of the experimental period, the animals were sacrificed under ether anesthesia, following overnight fasting, and blood samples were collected via intracardiac puncture using heparin and sodium citrate as anticoagulants. Whole blood was analyzed with an automated hematology analyzer (Abacus Junior Vet). Two blood films were prepared from each blood sample; one of the samples was stained with May Grunwald-Giemsa and examined for white blood cell differential count (22). The other sample was fixed in glutaraldehyde acetone fixative for α -naphthyl Acetate Esterase (ANAE) demonstration. The ANAE activity was determined from blood smears fixed in glutaraldehyde-acetone solution at -10 °C for 3 minutes, rinsed in distilled water, and then dried in the air. An incubation solution consisting of 20 mg of alpha naphthyl acetate substrate dissolved in 0.8 ml acetone, 4.8 ml of hexazotized pararosaniline ((hexazotization was performed by mixing equal volumes (2.4 ml each) of 4% sodium-nitrite and 2% pararosaniline) and 80 ml of buffered phosphate saline (pH 5). The final pH of the incubation solution was adjusted to 5.8 with 1N NaOH, and the solution was filtered. After two-hour incubation at 37°C, the smears were rinsed 3 times in distilled water, and the nuclei were stained for 20 minutes in 1% methyl-green prepared in acetate buffer (pH 4.2). Control specimens were prepared by incubating the smears in incubation solution without alpha naphthyl acetate (23).

The heparinized samples were centrifuged at 1700g for 15 min to separate the plasma and erythrocytes. Plasma was harvested by aspiration. Prior to analysis, erythrocytes were washed three times with an equal volume PBS (phosphate buffer solution).

Lipid Peroxidation Assay

Lipid peroxidation (as malondialdehyde, MDA) levels in plasma were determined using the method described by Yoshiko et al. (24),

based on the thiobarbituric-acid (TBA) reaction. The optical density was measured at 535 nm by spectrophotometer (Shimadzu UV 1208).

Antioxidant Enzyme and Molecules

Plasma ceruloplasmin level was determined using the method described by Colombo and Richerich (25). Phenylenediamine dichloride solution (7.95 mM, 2.5 mL) was added to all the test and blank tubes. Blank tubes were vortexed after adding a solution of 500 μ l of sodium azide and tubes were incubated at 37 ° C water bath for 15 minutes. After incubation, 500 μ l of sodium azide added to test tubes and absorbances were measured at 546 nm by spectrophotometer (Shimadzu UV 1208).

Whole blood glutathione (GSH) concentrations were determined using the method described by Beutler et al. (26). All of the non-protein sulfhydryl groups of red blood 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 erythrocytes was assayed by the decrease in absorbance of hydrogen peroxide at 240 nm as per the method of Aebi (27). Superoxide dismutase activity in erythrocytes was assayed spectrophotometrically as described by Sun et al. (28). 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 (Shimadzu UV 1208).

Lead, Copper and Zinc Estimation in Blood Samples

Blood samples were treated with concentrated nitric acid and centrifuged at 2500 rpm and then used for elemental analyses according to the methods of Alonson et al. (29) and Lai and Jamieson (30). The determination of elements was carried out using inductively coupled plasma atomic emission spectrometry (ICP-AES, Liberty Series-II Varian, USA). The wavelengths were 283.306, 213.856, 259.837 nm for lead, zinc and copper, respectively.

Statistical Analysis

The data were analyzed by one-factor ANOVA using the general linear models procedure of SAS (31). Software was the main effect of treatments. Differences between means were determined by Duncan's multiple range test at a significance level of $P < 0.05$.

Results

Total Phenolic Contents

Total phenolic content of pomegranate juice was estimated as 39.09 mmol/L (gallic acid equivalents).

Blood Lead and Mineral Levels

Figure 1 shows the lead, copper and zinc levels in the blood of the control, lead treated, and lead+PJ treated rats. The plasma lead level was lower in rats received pomegranate juice. On the other hand the plasma zinc and copper level were higher in rats received pomegranate juice.

Hematological Parameters

The erythrocyte and leukocyte parameters of the rats are shown in Table 1. The red blood cell (RBC) and white blood cell (WBC) counts, and the hemoglobin (Hb) and packed cell volume (PCV) values were significantly decreased in the rats exposed to lead compared to the controls ($p < 0.001$). In the rats received lead and either 30 or 60 μ l/day PJ (1050 and 2100 μ mol total phenolics/day, respectively), RBC, WBC count, Hb and PCV were significantly higher than in the rats exposed to lead alone ($p < 0.001$). Mean corpuscular volume (MCV) was slightly decreased, but not statistically significant in the rats exposed to lead; whereas this value in the rats that received lead and either level of PJ was intermediated between the values of control rats and the rats exposed to lead alone (Table 1).

The T lymphocytes contained 1-5 specific red brown ANAE positive granules (see Figure 2) in all groups. The ANAE activities in the rats exposed to lead were lower than those in the other groups. The highest ANAE activity was detected in the rats that received lead plus 60 μ l/day PJ.

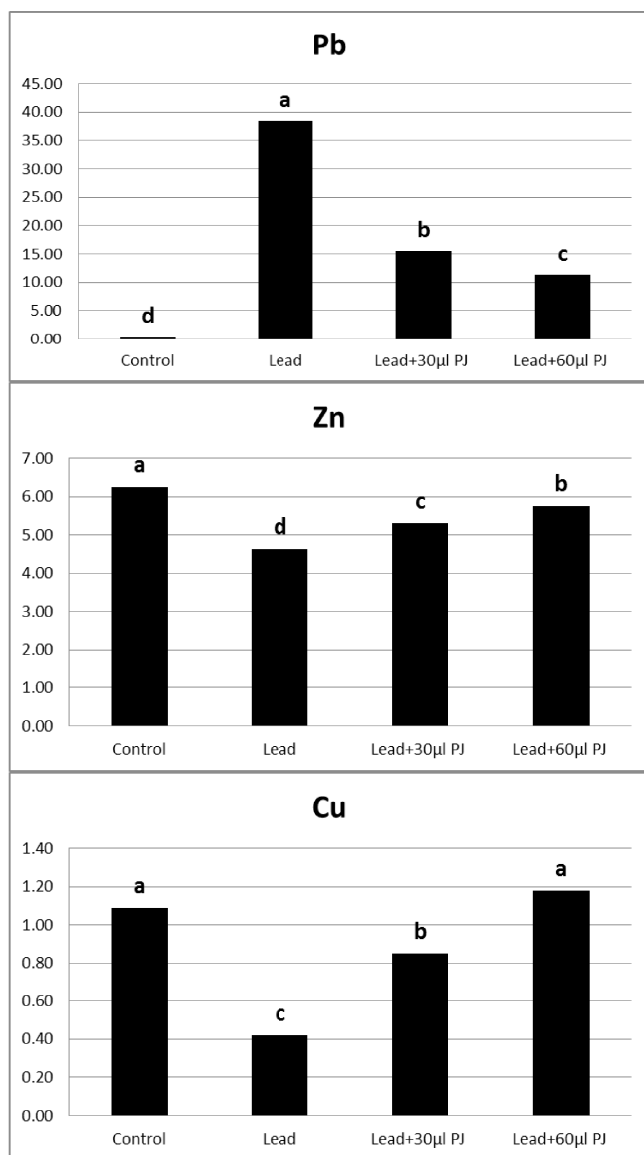


Figure 1. Plasma Pb, Zn, and Cu levels of experimental groups ($\mu\text{g/dl}$)

Oxidative Stress and Antioxidant Parameters

Table 2 shows selected parameters indicative of oxidative stress in rat RBCs. Lipid peroxidation, as determined by MDA formation, was highest in the rats exposed to lead ($39.17 \mu\text{mol/L}$) ($p < 0.001$). The MDA levels of the rats that received lead and either 30 or $60 \mu\text{l/day}$ PJ were 32.61 and

$28.80 \mu\text{mol/L}$, respectively. These values were significantly higher than the values obtained for control rats ($24.73 \mu\text{mol/L}$), but were significantly lower than the value obtained for rats exposed to lead alone ($p < 0.001$).

Erythrocyte GSH and plasma Cp levels, and erythrocyte SOD activity was decreased, while erythrocyte catalase activity was increased, in the group exposed to lead compared to the control. In contrast, these levels in the rats that received lead plus 30 or $60 \mu\text{l/day}$ PJ were close to the control values.

Discussion

Results from current study showed that phenolic compounds in pomegranate juice (PJ) had a strong protective effect against the lead-induced alterations in blood cells and oxidative stress markers. Results also indicated that these compounds decreased the lead level of blood while protected copper and zinc levels of blood. On the other hand, lead exposure enhanced the generation of reactive oxygen species (ROS) and lipid peroxidation and caused red blood cell damage and anemia.

The hematological system, brain, liver, kidney have been proposed to be important targets for lead-induced toxicity. The effects of lead on hematological system are thought to result in decreased HEM synthesis and, consequently, anemia (32). A protective effect of pomegranate juice on the hematologic system, in addition brain, liver, kidney and heart (un published data), was clearly evident in the current study. The detrimental effect of lead-induced lipid peroxidation on hematopoiesis, through disruption of HEM synthesis and shortening of the lifespan of

Table 1. Hematological parameters from whole blood analysis in the groups (n=10)

	Groups				Significance
	Control	Pb	Pb+30µl PJ	Pb+60µl PJ	
RBC	8.24±0.09 ^a	6.04±0.19 ^c	7.70±0.07 ^b	7.81 ±0.05 ^b	***
HB	13.31±0.20 ^a	9.84±0.17 ^b	12.86±0.14 ^a	13.05±0.19 ^a	***
PCV	37.62±0.43 ^a	29.25±0.94 ^b	36.93±0.32 ^a	37.37±0.18 ^a	***
MCV	45.65±0.29 ^b	48.61±1.54 ^a	47.99±0.77 ^{ab}	47.65±0.37 ^{ab}	NS
MCH	16.15±0.15	16.34±0.28	16,74±0.24	16.79±0.18	NS
MCHC	35.37±0.42	33.80±0.75	34.87±0.57	35.21±0.48	NS
WBC	13.10±0.87 ^a	7.63±0.41 ^c	9.91±0.5 ^b	10.00 ±0.58 ^b	***
LYM%	80.70±1.78	82.50±1.96	79.60±1.78	79.10±0.81	NS
NOTRO%	14.30±0.90	11.70±1.54	15.30±1.94	15.30±1.09	NS
MONO%	1.6±0.8	1.5±0.73	2.0±0.42	1.2±0.36	NS
EOZIN%	2,8±0,49	4,2±0,99	2,9±0,38	4,3±0,40	NS
BAZO%	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01	NS
ANAE (+) LYMP	66.6±1.81 ^b	51.5±2.08 ^c	65.3±3.33 ^b	79.4±1.65 ^a	

^{a-c} : Means values within a row having differing superscripts are significantly different by least significant differences test (p<0.05).
NS: non-significant, ***: p<0.001

Table 2. Selective oxidative stress parameters in red blood cells of the groups (n=10)

	Groups				P
	Control	Pb	Pb+30µl PJ	Pb+60µl PJ	
MDA(µmol/L)	24.73±0.74 ^d	39.17±1.31 ^a	32.61±0.50 ^b	28.80±0.53 ^c	***
Cp (mg/dl)	81.32±1.99 ^a	45.32±4.63 ^b	73.23±2.52 ^a	75.26±2.10 ^a	***
CAT (K/gHb)	54.25±2.52 ^c	226.64±17.14 ^a	101.59±8.34 ^b	77.98±4.87 ^{bc}	***
SOD (U/gHb)	1297.99±43.76 ^a	964.69±18.16 ^c	1019.79±16.40 ^{bc}	1089.74±16.95 ^b	***
GSH (mg/dl)	13.68±0.54 ^a	8.72±0.27 ^d	10.34±0.24 ^c	12.18±0.33 ^b	***

^{a-c} : Means values within a row having differing superscripts are significantly different by least significant differences test (p<0.05).
***: p<0.001

blood cells, was reduced in the rats that also received 30 or 60 µl/day PJ. Indeed, RBC, Hb, PCV values, and WBC and ANAE positive lymphocyte counts in these groups were higher than those in the rats exposed to lead alone. This can be attributed to the free radical scavenging activities of flavonoids and other plant phenolics or to the metal-binding activity and immune system stimulating properties of polyphenolics (11, 33). Polyphenols are multifunctional and can act as reducing agents, hydrogen donating antioxidants, and singlet oxygen quenchers. The antioxidant mechanism is

based on the donation of hydrogen and the formation of a phenoxyl radical, which then undergoes stabilization either by release of further hydrogen, or by reaction with another radical (34, 35). As expected that in this study, lead caused hemolysis and lipid peroxidation in red blood cells. While lead causes anemia by disrupting HEM synthesis, it also leads to oxidative stress due to increases in the production of superoxide radical, which also interacts with oxyhemoglobin (15).

Pomegranate juice, as a source of polyphenolic flavonoids, is rich in antioxidants in-

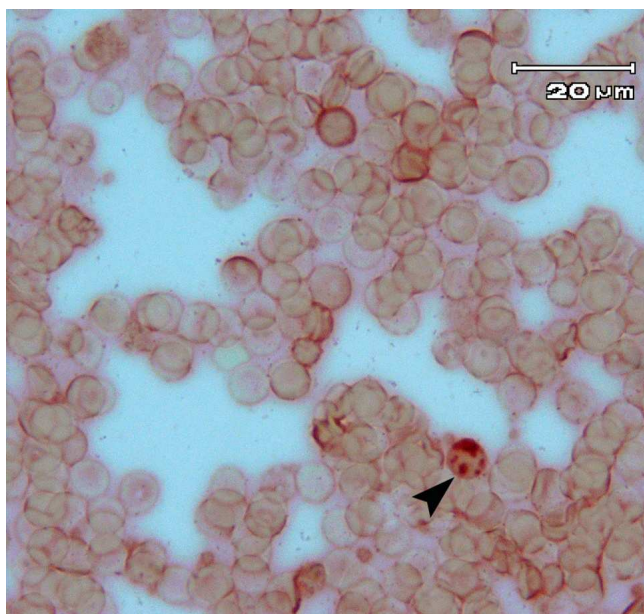


Figure 2. ANAE positive lymphocyte (arrow)

cluding tannins and anthocyanins. Polyphenols can interact with the surface of bilayers by adsorbing onto the polar head or the hydrophobic chains of lipids of cell membranes when they have hydrophobic and hydrophilic domains (24, 36) These interactions can result in functional changes of a number of membrane-associated events including the activity of membrane-associated enzymes, ligand-receptor interactions, ion and/or metabolite fluxes, and the modulation of signal transduction. Taking into consideration their antioxidant effects, polyphenols adsorbed onto a membrane surface could also provide a physical barrier to hydrosoluble radicals (37). When inserted into the lipid bilayer, polyphenols would be in close proximity to the sites of generation of lipid radical ($L\cdot$), lipid peroxy radical ($LOO\cdot$), and other lipid soluble radicals (37, 38) and could rapidly scavenge these as they form. In the current study, the findings for the rats that received pomegranate juice in addition to lead indicated that the phenolic compounds in pomegranate juice had very beneficial effects with respect to preventing/re-

ducing the oxidative damage caused by lead exposure. These findings were supported by the observed improvements in hematological values. Pomegranate juice did not completely prevent lead toxication, but it did significantly decrease the hemolysis caused by lipid peroxidation in red blood cell membranes.

In the present study, as expected, the increase in MDA levels and changes in antioxidant enzyme activities in rats exposed to lead is indicator of oxidative damage caused by lead. Lead increases the production of reactive oxygen species and also impairs the antioxidant defense systems. Lipid peroxidation as a consequence of reactive oxygen species causes cell death by disruption of cell membrane integrity (4). An improvement in antioxidant potential was observed in the rats that received PJ compared to the rats exposed to lead alone, as evidenced by decreased MDA levels.

On the other hand, positive changes observed in GSH and Cp levels, and SOD and CAT activities also showed that the polyphenols in pomegranate juice reduced the formation of cellular ROS and suppressed the detrimental effects of oxidative damage caused by lead.

Glutathione is the first line of defense against free radical induced damage. The tripeptide GSH contains reactive sulfhydryl groups ($-SH$) that protect the cells against oxidative stress by serving as a non-enzymatic antioxidant (39). The affinity of lead for $-SH$ groups may have caused changes in vital activities of cells by creating structural changes in membrane proteins, resulting in a decrease in $-SH$ groups of cells (40). GSH levels of the rats received pomegranate juice were higher than the rats received to lead alone in the current study (Table 2). Lead restricts the antioxidant activity of GSH by binding to the $-SH$ groups of GSH. On the other hand, an increase in GSH levels and decrease in blood lead level observed in the rats received pomegranate juice may be due to the metal-fixing activity of polyphenolic compounds. Phenolic compounds can be decreased the blood lead level by reducing the absorption of lead from

gastrointestinal tract and by limiting the retention of lead in metabolism. In fact, there is not enough research about that polyphenolic compounds has metal-binding properties that could replace the metal-binders. However, in the current study, as the decrease in blood lead level parallel to increase in blood zinc and copper level of the rats received pomegranate juice with lead, suggested that phenolic compounds reduced the negative effects of lead on trace element metabolism by reducing the absorption of lead from gastrointestinal tract and by limiting the retention of lead in metabolism. Metallothioneins are metal-binding proteins, which cysteine rich and low molecular weight. In fact, metallothioneins have protective effect against toxic metals. Although specific region for heavy metals-binding of metallothioneins is less, their expressions are increased in the presence of heavy metals such as cadmium, lead, and mercury (41). Metal-binding regions of metallothioneins have also specific areas for copper and zinc and it has been demonstrated that toxic metals can disrupt trace element metabolism (42). On the other hand metal binding capacity of metallothioneins is directly related to the metal density in cells (29). Therefore, the absorption of zinc and copper are reduced by lead-binded when lead density is more in cell. On the other hand, it is well know that polyphenols generate functional changes in membrane-bound enzyme activities (37). In this regard, polyphenols in pomegranate juice may be limited the lead absorption by preventing of the lead binding, as well as by generating functional changes in metallothioneins.

Superoxide dismutase and ceruloplasmin are important enzymatic and non-enzymatic antioxidants that play roles in protecting the cells against the detrimental effects of free radicals. Zinc and copper are required the activity of superoxide dismutase. The copper is involved in catalysis while the zinc is involved in the stability of the enzyme (43). Ceruloplasmin is also copper-glycoprotein that shows peroxidase activity (44). An increase both of SOD activity and ceruloplasmine level of the rats re-

ceived PJ with lead may be related to the increase of these minerals levels.

Catalase activity increased despite a decline in the other antioxidant defense systems examined in the rats received PJ and lead. The increase observed in erythrocyte catalase activity of the rats exposed to lead may be a compensation mechanism to counteract the decreasing activity of SOD and the levels of Cp and glutathione. In fact, oxidative stress caused by lead increases production of ROS. Catalase plays an important role in scavenging hydrogen peroxide (H_2O_2) which is one of reactive oxygen species (4). Hence, the increase in catalase activity could also be attributed to a defense mechanism generated by the red blood cells against an increased level of H_2O_2 caused by lead.

As a result, the current study presents evidence that the phenolic compounds, flavonoids, and antioxidant activities of pomegranate juice provides effectively protection in a hematological system against the changes and oxidative damage caused by lead. Limiting effect of polyphenols on lead retention in metabolism and its tendency for reduce on the detrimental effects generated by lead in trace mineral (Cu and Zn) metabolism may be related to the functional changes in metallothioneins generated by polyphenols. Further studies should clarify about the functional changes in metallothioneins generated by polyphenols.

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Conflict of interest declaration

Authors declare no conflict of interests.

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