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Possible protective role of punicalagin on oxidative stress, inflammation and genotoxicity in ethanol-induced liver toxicity

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ABSTRACT: Alcohol consumption is a tradition in most cultures but are increasingly being arised as possessing a potential for misuse. Punicalagin is a phenolic compound that is found in forms alpha and beta in pomegranates. In this study, the protective role of punicalagin on oxidative stress, inflammation and DNA damage caused by ethanol (EtOH) in liver tissue were examined. Wistar albino rats were divided into 4 groups as control, eth, punicalagin, EtOH EtOH + punicalagin. In EtOH groups, rats were treated with EtOH (4g/kg) for 21 days, in punicalagin groups, rats were treated with punicalagin (4 mg/kg) for 21 days. In the liver tissue, superoxide dismutase (SOD), catalase (CAT) activities and malondialdehyde (MDA) and glutathione (GSH) levels, in serum AST, ALT, LDH activities and TNF-α, IL-6 levels were measured. Genotoxicity was evaluated using comet assay. Based on these experimental results, while EtOH increased ALT, AST and LDH enzyme activies and induced inflammation and oxidative stress. Punicalagin reduced IL-6, TNF-α, MDA levels, ALT, AST, LDH enzyme activities and increased SOD, CAT activities and GSH levels. EtOH significantly increased the percentage of damaged cells (type II, III and IV) and genetic damage index compared to the other groups (control, punicalagin and EtOH +punicalagin). Punicalagin was not genotoxic compared to the control. Furthermore, punicalagin reduced the genotoxic effect, induced by EtOH, with the sharp decrease in damaged cells (from 14.00±1.22 to 2.20±1.30) and genetic damage index (from 1.20±0.05 to 0.14±0.05). Punicalagin has antioxidant, anti-inflammatory and protective role against to ethanol induced liver toxicity.

KEYWORDS: Punicalagin; liver toxicity; oxidative stress; genotoxicity; ethanol.

1. INTRODUCTION

Alcohol consumption is a tradition in most cultures but are increasingly being arised as possessing a potential for misuse. Approximately, 3% to 12% of fatty liver or hepatitis caused by binge drinking develop into cirrhosis annually with increasing mortality. It has been known that there is a positive correlation between chronic alcohol consumption and liver diseases [1,2]. Daily 80 grams or more alcohol consumption is considered a risk to human health [3]. Although liver damage can occur as a result of various factors such as genetic and environmental, including chronic alcohol consumption. One of the key mechanisms is oxidative stress. Oxidative stress is the imbalance between antioxidants and oxidants. Small amount reactive oxygen species (ROS) are produced by natural process of metabolism in cell organisms, especially in mitochondria. Inflammation, aging, ischemia, radiation and ethanol exposure induce overproduction of ROS which is damage proteins and DNA of hepatocytes [4-6]. Chronic ethanol comsuption induce the production of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 from kuppfer cells in the liver. These cytokines are induced in alcohol-induced liver toxicity and alcohol-related liver diseases [7]. *Punica granatum* (pomegranate) is a species belonging to the family of punicaceae and can be used in the treatment of several diseases [8]. Punicalagin a kind of phytochemicals isolated from pomegranate fruit, has diverse pharmacological properties, such as antioxidant, antimicrobial, anti-inflammatory. The aim of the current study is to explore the antioxidant, anti inflammatory effect of punicalagin in ethanol-induced liver toxicity in rats.

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2. RESULTS

SOD activity in EtOH and EtOH +Pun groups was reduced by 60%, 39.13%, respectively compared to control group. These decreases were significant ($p < 0.05$) but there was no significant difference between the Pun group. In the EtOH +Pun group, SOD activity was higher than EtOH group ($p < 0.05$) (Figure 1).

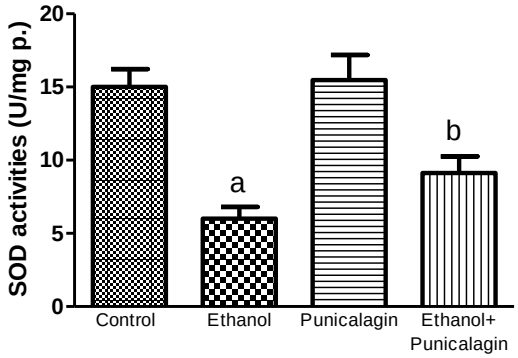

Figure 1. SOD activities in liver tissue. [a] Significant lower than the control groups ($p<0.05$). [b] Significant higher than the ethanol groups ($p<0.05$).

CAT activity in EtOH and EtOH +Pun groups decreased by 67.37%, 22.43%, respectively when compared to control group. These decreases were significant ($p < 0.05$) but there was no significant difference between the Pun group. In the EtOH +Pun group, CAT activity was higher than EtOH group ($p < 0.05$) (Figure 2).

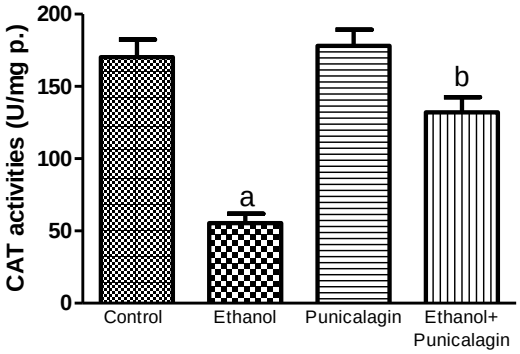

Figure 2. Catalase activities in liver tissue. [a] Significant lower than the control groups ($p<0.05$). [b] Significant higher than the ethanol groups ($p<0.05$).

In the EtOH and EtOH +Pun groups, GSH levels reduced by 40%, 12.72%, respectively compared to control group. These decreases were significant ($p < 0.05$). In the EtOH +Pun group, GSH levels were higher than EtOH group ($p < 0.05$) (Figure 3).

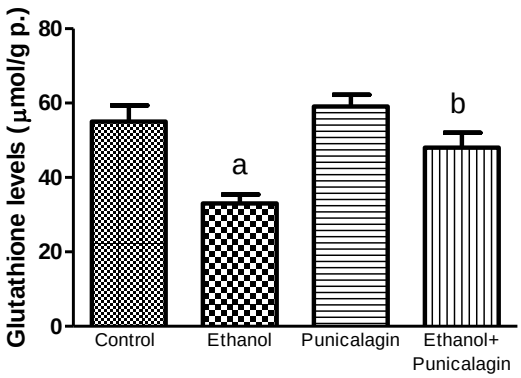

Figure 3. Glutathione levels in liver tissue. [a] Significant lower than the control groups ($p<0.05$). [b] Significant higher than the ethanol groups ($p<0.05$).

MDA levels in EtOH and EtOH +Pun groups were raised by 51.34%, 17.49%, respectively compared to control group. These raises were significant ($p<0.05$). In the EtOH +Pun group, MDA levels were lower than EtOH group ($p <0.05$) (Figure 4).

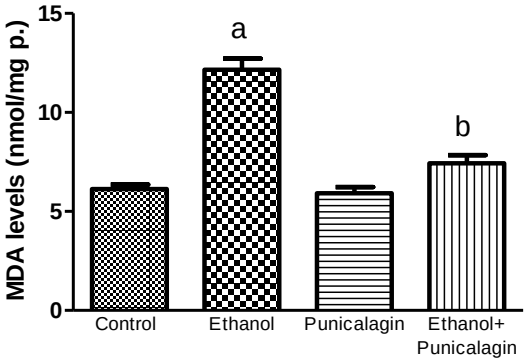

Figure 4. MDA levels in liver tissue. [a] Significant higher than the control groups ($p<0.05$). [b] Significant lower than the ethanol groups ($p<0.05$).

ALT levels in EtOH and EtOH +Pun groups were increased by 67.3%, 33.06% respectively compared to control group. These increases were significant ($p<0.05$) and AST levels in EtOH and EtOH +Pun groups were raised by 36.28%, 27.58%, respectively compared to control groups. These raises were significant ($p<0.05$). In the EtOH +Pun group, AST and ALT levels were lower than EtOH group ($p <0.05$) (Figure 5).

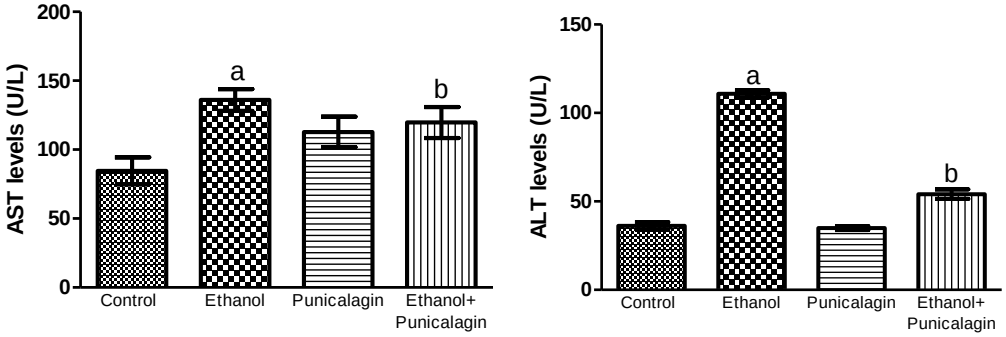

Figure 5. ALT and AST levels in serum.[a] Significant lower than the control groups ($p<0.05$). [b] Significant higher than the ethanol groups ($p<0.05$).

LDH levels in EtOH and EtOH +Pun groups were increased by 82.87%, 67.56%, respectively compared to control groups. These raises were significant ($p<0.05$). In the EtOH +Pun group, LDH levels were lower than EtOH group ($p <0.05$) (Figure 6).

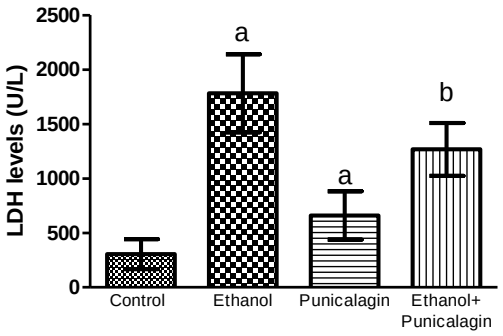

Figure 6. LDH levels in serum. [a] Significant higher than the control groups ($p<0.05$). [b] Significant lower than the ethanol groups ($p<0.05$).

In the EtOH and EtOH +Pun groups, IL-6 levels raised by 52.27%, 46.83%, respectively compared to control group. These raises were significant ($p < 0.05$) and TNF-α levels in EtOH and EtOH +Pun groups increased by 60%, 33.33%, respectively compared to control group. These raises were significant ($p < 0.05$). In the EtOH +Pun group, TNF-α and IL-6 levels were lower than EtOH group ($p < 0.05$) (Figure 7).

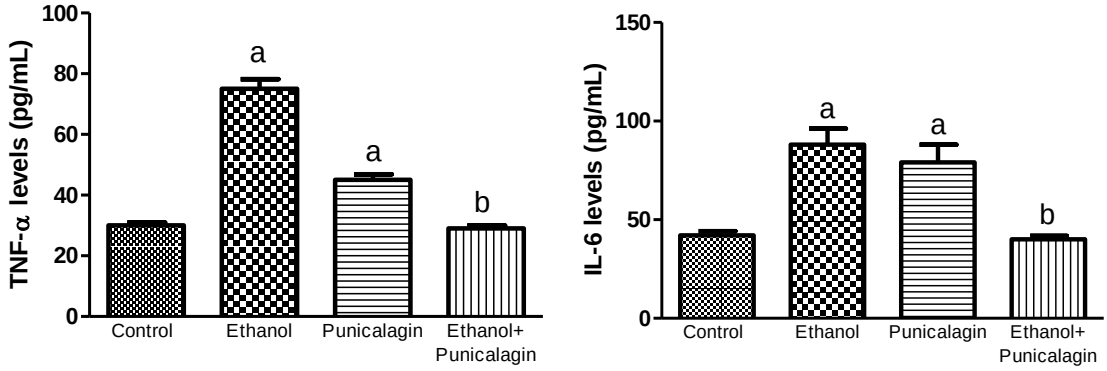

Figure 7. TNF-α and IL-6 levels in serum. [a] Significant higher than the control groups ($p<0.05$). [b] Significant lower than the ethanol groups ($p<0.05$).

The frequency of DNA strand breaks in blood cells of ethanol/punicalagin treated rats are shown in Table 1 and Figure 8 shows the DNA strand breaks in blood cells of control, ethanol and punicalagin treated rats. Table 1 shows that following ethanol treated, the main tail lengths were significant increases compared with the control group. When rats were exposed to punicalagin, a significant increase of DNA strand breaks was not detected. Also, no change was measured among the control group and the punicalagin treated group. After punicalagin treated, punicalagin was shown strong protective activity against the ethanol–induced DNA strand breaks.

Table 1. Analysis of DNA damage as measured by comet assay in rat blood cells.

Treatment groups	Proportion of damaged nuclei (%) [a]					% of Damaged cells (II+III+IV)[b]	Genetic damage index (GDI)[c]
	Type 0	Type I	Type II	Type III	Type IV		
Ethanol	7.8	78.2	4.4	5.2	4.4	14.00±1.22*	1.20±0.05*
Punicalagin	97.6	1.6	0.2	0.2	0.4	0.80±0.84	0.04±0.03
Ethanol+Punicalagin	90	7.8	1.4	0.2	0.6	2.20±1.30	0.14±0.05
Positive Control	0	19	54.4	25.8	0.8	81.00±4,18*	2.08±0.13*
Negative Control	96.2	2.8	0	0.4	0.6	1.00±0	0.06±0.01

*P<0.001
[a] 0-IV indicate grades of DNA damage [16]
[b] Percentage of damaged cells = Type II+III+ IV [17]
[c] GDI, Genetic damage index = (Type I+2*Type II+3*Type III+4*Type IV)/ (Type 0+I+II+III+IV) [18].

3. DISCUSSION

The formation of reactive oxygen species and other free radicals is the result of physiological processes such as cellular redox in biological systems. Free radicals can be removed by antioxidant defense systems in the body [9,10]. Oxidative stress is a phenomenon caused by balance shifts towards free radicals in case of increasing free radical production or inadequate antioxidant defense systems. It can damage to cell, lipid, protein, and cause DNA damage [11,21]. Antioxidant defense systems were found to be insufficient as a result of increased formation of free radical and lipid peroxidation products in diseases such as diabetes, heart diseases, rheumatoid arthritis, muscle, skin, eye diseases, hypertension, psoriasis, cancer and liver diseases [13-15].

In our study, ethyl alcohol was used for *in vivo* liver toxiticy model [16-20]. Hepatotocytes have enzymatic and non enzymatic defence system. Among antioxidant enzymes superoxide dismutase, catalase, GSH-Px are well studied. The function of SOD is to protect against the adverse effects of superoxide radicals.

It converts superoxide anion to hydrogen peroxide and oxygen. SOD enzyme activity is higher in tissues with intensive oxygen use and enzyme activity increases in direct proportion to oxidative stress [21, 22]. The hydrogen peroxide is converted to O_2 and H_2O by catalase enzyme [33]. At high concentrations of hydrogen peroxide, the catalase enzyme is highly active [24]. Hydrogen peroxide is removed by CAT and glutathione peroxidase enzyme activity [20].

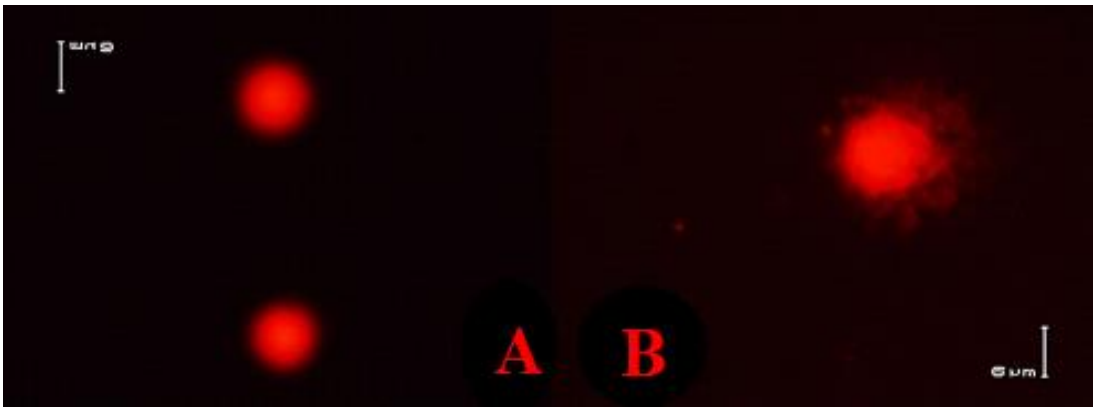

Figure 8. Undamaged (A) and damaged (B) blood cells of rats.

Glutathione has a critical role in antioxidant defence system against free radicals [25]. Reduced glutathione is a powerful antioxidant, reacting rapidly with lipid peroxidation products that occur as a natural consequence of free radical growth. [26, 27].

Previous studies have emphasized that the EtOH administration led to increased the formation of malondialdehyde and depletion of superoxide dismutase, catalase and glutathione peroxidase in liver tissue [28]. The SOD, CAT activities and GSH levels were measured to evaluate the antioxidant effect of punicalagin in ethanol induced oxidative stress in liver tissue. Daily ethanol administration caused a significant decrease in the SOD, CAT activities and GSH levels as compared to the control rats. The SOD, CAT activities and GSH levels were increased significantly with the treatment of Punicalagin at 4 mg/kg. LDH, ALT and AST are liver damage marker and released by hepatocytes in case of liver abnormalities.

Ethanol toxicity significantly increased ALT, AST and LDH activities as compared to control. Interestingly, treatment with punicalagin caused significantly decline in the liver enzyme such as ALT, AST and LDH. Treatment with Ethanol (4g/kg/day) for 21days increased ALT, AST levels in rats. These findings are in agreement with previous study [29]. Decreased defensive non-enzymatic and enzymatic antioxidants in liver cells results in mitochondrial dysfunctions and increased lipid peroxidation, which can leads to the liver necrosis [20]. Lipid hydroperoxides are formed by reaction of unsaturated fatty acids in membrane phospholipids with oxygen, this reaction is called lipid peroxidation reaction. Cholesterol and unsaturated phospholipids in the cell membran react easily and quickly with free radicals leads to lipid peroxidation [30, 31]. The MDA levels in ethanol group were significantly increased. However punicalagin treatment decreased significantly MDA levels. ROS and endotoxins formed after chronic ethanol consumption stimulate the expression of proinflammatory cytokines through NF-κB. Activated T-cells and macrophages in alcoholic hepatitis secrete cytokines containing TNF-α and IL-6 which have an important role in maintaining ethanol-mediated liver injury [32]. TNF-α and IL-6 levels were significantly increased in ethanol treated group. Punicalagin treatment reduced TNF-α and IL-6 levels. It has been shown that 4mg/kg punicalagin decreased mRNA levels of TNF-α, IL-18, and IL-1β in inflammatory bowel disease [33].

Acetaldehyde, which is formed as a result of ethanol metabolism, can cause the formation of free radicals. Therefore, oxidative stress and inflammation processes can initiated [34]. According to several studies ethanol exposure increase free radical production and the frequency of DNA strand breaks [35]. The antioxidant effect of punicalagin has been investigated in the rat liver and it have protective effect in the rat liver. The concentration of ethanol tested significant increased the DNA strand breaks, as indicated by comet assay. The percentage of damaged cells (type II, III, IV) at ethanol group elevated from 1 to 14.00±1.22 compared to the control. In addition, genetic damage index increased from 0.06±0.01 to 1.20±0.05. The protective effect of punicalagin significantly reduced the genotoxic effect induced by ethanol. Overall, our study demonstrated that the antioxidant effect of punicalagin prevented the DNA strand breaks on the rat blood cells. However, more studies might be conducted to better understand molecular pathways underlying the protective effect of punicalagin on various genotoxic agents.

4. CONCLUSION

As a result of our study, it can be said that punicalagine can be used as an alternative molecule in the treatment of diseases that may occur due to alcohol toxicity.

5. MATERIALS AND METHODS

5.1. Animals and experimental procedure

Punicalagin was obtained from Santa Cruz Biotechnology Inc. (Dallas, TX, USA) (CAS 65995-63-3). 24 adult female Wistar albino rats (250-300 g) were used in the experiments, which were maintained 12 hour dark-light cycle. Rats were obtained from the Experimental Medicine Research Center of Mersin University Faculty of Medicine. Experimental animals divided into 4 groups randomly. Group 1: Control group (rats were treated with serum physiologic for 21 day); Group 2: Ethanol group (rats were treated with 4 g/kg ethanol for 21 day); Group 3: Punicalagin group (rats were treated with 4 mg/kg punicalagin for 21 day); Group 4: Punicalagin+ethanol group (rats were treated with 4 mg/kg punicalagin + 4 g/kg ethanol for 21 day). The groups were formed by taking into account that the total weight of the rats was approximately the same. Each group was fed with standard rat feed in separate cages. During the experiment, drinking water was changed and cage was cleaned every other day. Liver tissues of animals was isolated under anesthesia (ketamine 200 mg/kg i.p.) than the tissues were passed through saline solution and the tissues were stored at -80°C until experiment day.

In this study, approval was obtained from Mersin University Animal Experiments Local Ethics Committee for all experimental procedures performed on rats (2017/ HADYEK/24). All procedures were performed according to NIH Guidelines for Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

5.2. Measurement of biochemical parameters

Serum levels of ALT, AST and LDH were detected colorimetrically using commercially available kits by AU5800 (Beckman Coulter, USA) autoanalyzer according to the manufacturer's instructions.

5.2.1. MDA level and antioxidant enzyme activities

Liver tissues were weighed 100 mg wet weight and homogenized by adding physiological saline solution to a ratio of 1/10 (w/v). The tissue samples transferred to glass tubes and centrifuged at 13,000 rpm for 10 minutes at +4°C.

Malondialdehyde (MDA) levels were measured by thiobarbituric acid reaction via spectrophotometer at 532 nm. Catalase (CAT), superoxide dismutase (SOD) enzyme activities and glutathione (GSH) levels were measured as antioxidant enzyme activity. GSH levels were determined according to Sedlak and Lindsay method [36].

Determination of CAT enzyme activity was carried out according to the method of Aebi [37]. Determination of SOD enzyme activity is based on the measurement of the color produced by the reaction of superoxide radicals formed by xanthine oxidase in the presence of xanthine with nitroblue tetrazolium (NBT) via spectrophotometer at 560 nm [38].

5.2.2. Measurement of cytokine levels

Tumor necrosis factor- α (TNF-α) and Interleukin-6 (IL-6) levels were measured for each 100 µL serum sample by using ELISA kit (Catalog Number: MBS282960 and MBS355410, respectively, My BioSource, Inc., San Diego, CA, USA) according to manufacturer's instructions.

5.2.3. Liver tissue protein content

Total protein content in liver tissue homogenates was measured by the Lowry method [39]. Bovine serum albumin was used as standard.

5.3. Blood sample collection and preparation of slides for the comet assay

At the end of this exposure period, a whole blood samples were collected from the cardiac of each animal in syringaes containing heparin. The collected blood samples were used to evaluate DNA damage in the blood cells. The DNA damage was demonstrated using the comet assay that used to measure the DNA

strand breaks in individual cells. The alkaline version of the comet assay was performed as described by literature method, with a minor modification [40]. Cell viability determined by trypan blue exclusion assay.

Slides were prepared in duplicate per sample per experiment. Briefly, pre-cleaned glass microscope slides were coated with 0,5% normal melting point agarose in PBS (Ca2+ and Mg+2 free). Then, 30 µl of heparinized blood cells were mixed with 250 µl of 0,65% low melting point agarose in PBS. 100 µl of the cell suspension was rapidly added to pre-coated microscope slides. The prepared slides were conserved at +4°C for 30 min. Subsequently, the slides were immersed in cold lysis solution for 1 h at +4°C in dark. After the lysis, the slides were incubated for 15 min in a horizontal electrophoresis tank fulled with cold electrophoresis solution. Following incubated, electrophoresis was performed [300 mA, 25 V, 20 min] and the slides were neutralized. The slides were stained with ethidium bromide (20 µg/ml). One hundred cells on each slide were analysed and were scored using a fluorescence microscope.

5.4. Statistical analyses

SPSS 25 (Statistical Package for Social Scicences) (IBM Corporation) package program was used for statistical analysis. One-sample Kolmogorov-Smirnov test was used to determine whether the results in the groups had normal distribution or not. Data were expressed as mean ± standard deviation, and p values of 0.05 or less were considered statistically significant. One-way analysis of variance (ANOVA) and Tukey tests were used for the evaluation of biochemical parameters.

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Conflict of interest statement: The authors declared no conflict of interest

Ethical committee aproval: All experimental procedures were approved by Mersin University Local Animal Ethics Committee (Decision number: 2017/HADYEK/24) and performed in compliance with national and local animal care and use guidelines.

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