Slov Vet Res
DOI 10.26873/SVR-2056-2024
UDC 599.322/.324:57.018.634:591.463.2:633.93:634.64
Pages: 83–93

Original Research Article

Pomegranate Juice Treatment Prevents Carbon Tetrachloride(CCl4)-induced Testicular Damage in Rats: A Biochemical and Histological Study

Key words

CCl4 toxicity;
infertility;
pomegranate juice;
carboxylesterase;
oxidative stress;
histopathology

Ömer Faruk Kalkan[1], Ahmet Türk[2], Cihan Çitil[3], Miraç Uçkun[4], Ahmet Özkaya[5], Ertan Yoloğlu[6], Yusuf Özay[7], Şeyda Çağri Bülbül[8], Nadir Bilgin Akgül[9], Zafer Şahin[1], Akın Kirbaş[10]

[1]Karadeniz Technical University, Faculty of Medicine, Department of Physiology, Trabzon, [2]Department of Histology and Embryology, Faculty of Medicine, Adiyaman University, Adiyaman, [3]Department of Biology, Faculty of Science and Art, Çankırı Karatekin University, Çankırı, [4]Department of Food Engineering, Faculty of Engineering, Adiyaman University, Adiyaman, [5]Department of Chemistry and Chemical Processing Techniques, Vocational School of Technical Sciences, Adıyaman University, Adıyaman, [6]Department of Science Education, Faculty of Education, Adiyaman University, Adiyaman, [7]Department of Medical Biology and Genetics, Faculty of Medicine, Adiyaman University, Adiyaman, [8]Department of Chemistry, Faculty of Science and Art, Adiyaman University, Adiyaman, [9]Department of Medical Biochemistry, Medical School, İnönü University, Malatya, [10]Faculty of Veterinary Medicine, Veterinary Internal Diseases, Atatürk University, Erzurum, Türkiye

*__Corresponding author:__ ofkalkan@ktu.edu.tr

Abstract: We investigated the side effects of carbon tetrachloride (CCl4) on testicular tissue and explored the protective effects of pomegranate juice (PJ) against CCl4 exposure. Twenty-eight male Wistar albino rats were randomly divided into four groups as follows: Control, PJ, CCl4, and CCl4+PJ. We assessed serum testosterone levels and evaluated carboxylesterase (CaE), malondialdehyde (MDA), glutathione (GSH), glutathione reductase (GR) and glutathione Stransferase (GST) parameters. Histopathological changes were examined using CD68 immunoreactivity to detect macrophage activity. Analysis revealed a significant increase in CaE activity in the PJ group that compared to the control group ($p < 0.05$). CCl4 exposure reduced CaE activity, which was partially restored by PJ in the CCl4+PJ group ($p < 0.05$). PJ also significantly reduced the elevated MDA levels induced by CCl4 ($p < 0.05$). CCl4 alone decreased the reduced glutathione (GSH) levels ($p < 0.05$), however PJ had no effect on GSH levels ($p > 0.05$). Neither CCl4 nor PJ had an effect on glutathione reductase (GR) and glutathione S-transferase (GST) activity ($p > 0.05$). In conclusion, PJ reversed histological damage and restored reduced testosterone levels. Furthermore, it alleviated CCl4-induced oxidative stress by utilizing an alternative antioxidant system rather than glutathione.

Received: 22 May 2024
Accepted: 28 April 2025

Introduction

Male infertility is a significant global health problem that affects couples, and its incidence is increasing year by year. An estimated more than 48 million couples worldwide are affected by infertility problems (1). The most important parameters affecting male fertility are endocrine and testicular factors. Various factors, including hormonal disorders, physical ailments, lifestyle choices, and psychological issues, contribute to male infertility (2). Additionally, environmental factors and chemical pollutants can also damage the male reproductive and endocrine systems, leading to infertility (3). Carbon tetrachloride (CCl4) also known as tetra chloromethane is a non-flammable, dense, colourless chemical, is a toxic substance that can damage the liver, central nervous system, and reproductive system (4). It was utilized in the manufacture of refrigeration fluids and aerosol can propellants, as a pesticide, cleaning fluid, and degreasing agent, as well as in fire extinguishers and spot removers. Due to its harmful health effects, the use of CCl4 is restricted. However, individuals may still be exposed to CCl4 in the air through accidental releases during production and use, as well as from its disposal in landfills where it can evaporate into the air or leach into groundwater (5). Exposure to CCl4 results in oxidative stress within testicular tissues, causing a decrease in the activity of antioxidant enzymes. CCl4 has been demonstrated to induce oxidative damage through the generation of free radicals and an increase in lipid peroxidation, which are primary indicators of oxidative stress. The formation of free radicals is heightened in tissues exposed to CCl4 (6). Consequently, the depletion of antioxidant mechanisms within cells disrupts the pro-oxidantantioxidant balance, leading to tissue damage (7). This complex tissue damage process impairs testicular function by affecting testicular histology and antioxidant system (8) and also CCl4 intoxication leads to a decrease in androgen levels (9). Numerous studies have investigated the use of specific antioxidant molecules for preservation or therapeutic purposes. (10, 11).

Furthermore, it is also important to assess the actual effect of such remedies in order to avoid bogus claims about the health benefits of (semi-)natural products. Although there are several pharmacological treatments available for infertility, they often come with numerous side effects and occasionally may prove ineffective (12). Therefore, consuming natural products is an alternative way to mitigate the chronic effects of chemical pollutants on reproductive function by reducing risk factors (13).

Pomegranate juice (PJ) is a natural product abundant in various phytochemicals, which exhibit a diverse array of biological activities, including anti-oxidative, anti-inflammatory, and antiapoptotic properties. These compounds are pivotal in the oxidation of unsaturated fatty acids and the neutralization of free radicals, rendering PJ a highly beneficial natural product for health. With its longstanding history of safe usage in both traditional and modern medicine, PJ boasts significant antioxidant capacity (14). Numerous studies have demonstrated the protective effects of pomegranate phytochemicals on the reproductive system (15, 16). However, there is limited data regarding its potential protective role against CCl4-induced toxicity.

In current study we investigated the potential protective efficacy of PJ on reproductive system induced by CCl4 in rats. Biochemical markers (carboxylesterase (CaE), malondialdehyde (MDA), glutathione (GSH), glutathione reductase (GR) and glutathione S-transferase (GST) elevated to detect the antioxidant pathway) endocrine (testosterone levels) and histological parameters have been examined to detect the possible protective effect of PJ against CCl4 induced damage.

Material and methods

Chemical composition of pomegranate juice

The content of PJ was determined as follows: phenolic acid at 490.75 mg/kg, anthocyanin at 137.1 mg/L, ellagic acid at 175 mg/100 g, total flavonoids at 63 mg/kg, and total antioxidants at 1530 mg/kg.

Preparation of pomegranate juice

Pomegranates sourced freshly from Adıyaman, Turkey, underwent meticulous preparation. Following a thorough wash and drain, they were halved and blended using an electric blender. The resulting juice was subsequently stored in 1 ml portions at -20°C until required. We used frozen juice for one week, then switched to fresh juice for further experiments.

Animals and experimental protocol

The research was carried out at the Adıyaman University Animal Experiment Centre, with ethical clearance granted by the Adıyaman University Animal Experiments Centre Ethics Committee, protocol number 2022/17. Twenty-eight adult male Wistar albino rats (11-12 weeks old and weighing between 240–260 g), were included in the study. These rats were housed under controlled conditions at a room temperature of 22 ± 2°C, with a 12-hour light-dark cycle, and provided with unrestricted access to food (a standard laboratory diet (RT-FR-01, DSA Agrifood Products INC, Kırıkkale, Turkey) and water.

The animals were randomly divided into four groups (same aged rats selected from different cages, maximum 2 animals from same cage). Before the experimental process, the rats were housed in cages with 4 animals per cage for four weeks. During the experimental phase, the rats were randomly distributed into groups of 7 animals each. The 7 rats in each group were then divided into cages with 3 and 4 animals per cage. The rats were housed in cages measuring 90 cm (L) x 60 cm (W) x 120 cm (H). Groups has been as follows: Control group, CCl4 group, PJ group, and CCl4+PJ group. Prior to any experimental procedures, the animals were housed in cages without any experimental interventions for adaptation. Following the adaptation period, experimental procedures were carried out as described below:

Control group: received the solvent of CCl4, olive oil, twice a week (i.p.) for a duration of 6 weeks, followed by saline (4 ml/kg) every other day for the succeeding 30 days.

CCl4 group: was administered CCl4 (Bayer) prepared in 50% olive oil via intraperitoneal injection (i.p.) at a dosage of 0.2 ml/100 g, twice a week. Rats received CCl4 (0.2 ml/100 g) over a period of six weeks (17).

PJ group: pomegranate juice was administered to the animals via an orogastric tube at a dosage of 4 ml/kg per day for a duration of 30 days (18).

CCl4+PJ group: the animals were injected i.p. with CCl4 at a dosage of 0.2 ml/100 g twice a week for 6 weeks. Subsequently, for the following 30 days, pomegranate juice was administered to the animals via an orogastric tube at a dosage of 4 ml/kg per day.

Sample collection

After the 6 weeks of experimental period, the animals were euthanized one day after the last application by decapitation. Blood samples were obtained from the cranial vena cava using a sterile injector containing heparin. The samples were then centrifuged at 3000g for 5 minutes to separate the plasma. The plasma was subsequently isolated and stored at -20°C until further hormonal analyses. Testes, epididymis, seminal vesicles, and ventral prostate were removed and cleared from adhering connective tissue. Right testis tissues were kept at –20°C for biochemical analysis and left testis tissues were fixed in 10% neutral formalin solution for subsequent histological examinations.

Homogenization of tissue samples

To determine the levels of MDA, GR, GSH, GST and CaE tissue homogenization was conducted using a Potter–Elvehjem glass homogenizer, utilizing an ice-cooled 0.1 M Kphosphate buffer containing 0.15 M KCl, 1 mM EDTA, and 1 mM DTT in a 1:4 ratio of total tissue weight (w/v). Following homogenization, the homogenates were transferred to Eppendorf tubes and subjected to centrifugation for 20 minutes at 16, 000 g at 4°C (using a Sigma 2-16 K centrifuge, St. Louis, Missouri). Following centrifugation, the supernatant fraction was collected for subsequent analysis.

Biochemical analysis

Determination of MDA and reduced GSH levels

MDA and reduced GSH levels were evaluated using a microplate reader spectrophotometer system (Thermo TM Varioskan Flash, Thermo Scientific). Testicular MDA levels were determined by assessing the relative production of reactive substances of thiobarbituric acid (19). The results were presented as nmol/g wet tissue weight. Reduced GSH activity was measured by its reaction with DTNB to form a compound absorbing at 412 nm (20). The results were expressed as nmol/mg wet weight tissue.

Determination of glutathione s-transferase (GST) activity

To measure GST activity, a solution of 20 mM 1-chloro-2.4 dinitrobenzene (CDNB) was initially prepared in 96% ethanol, which served as the substrate. Reductive glutathione (0.002 M) was utilized as the cofactor in the reaction (21). Briefly, 10 µl of supernatant, 100 µl of phosphate buffer (0.1 M, pH 6.5), 100 µl of the GSH mixture, and finally 10 µl of CDNB were transferred into the microplate wells. These were then inserted into the microplate reader system, and the absorbance change was recorded at 344 nm for 2 minutes at 25°C. Specific GST activity was computed as nmol/min/mg protein.

Determination of glutathione reductase (GR)

The analysis of GR activity was conducted using a modified method (22). The assay solution consisted of 50 mM Tris–HCl buffer (pH 8.0), 1 mM EDTA, 1 mM GSSG, and 0.1 mM NADPH. One enzyme unit was defined as the quantity that oxidizes 1 µmol of NADPH per minute under the specified assay conditions. GR activity was computed as nmol/mg protein.

Determination of carboxylesterase (CaE) activity

The previously described spectrophotometric methods were adapted for the analysis of CaE activity using a microplate reader system (23, 24). In the activity assay, p-nitrophenol acetate (PNPA) was dissolved in 26 mM 96% ethanol to serve as the substrate. A reaction solution comprising 5 ml of sample and 250 ml of 0.1 mM Trizma buffer (pH 7.4) was incubated for 3 minutes at 25°C. The reaction was initiated by adding 5 ml of the substrate to the reaction solution. Changes in absorbance were monitored at 405 nm for 2 minutes at 25°C. Specific CaE activity was calculated as nmol/min/mg protein.

Analysis of serum testosterone levels

Testosterone levels were assessed using a commercial rat ELISA kit (Beckman Coulter, Inc., USA). The inter- and intra- assay coefficients of variation were 5.6%–6.8% and 2.9%–4%, respectively. Testosterone levels were expressed as ng/mL.

Histopathological evaluation

Preparation of Tissues and Hematoxylin & Eosin Staining

Testicular tissues were preserved in a 10% neutral formalin solution at room temperature until the day of analysis for histological examination. To perform light microscopy analyses, routine tissue processing was conducted using an automatic tissue processor (Leica TP1020, Nussloch, Germany), and 4-6 µm thick sections were obtained from the prepared paraffin blocks using a Thermo Shandon Finesse ME microtome (Thermo Fisher Scientific, Cheshire, UK). The slides prepared with Hematoxylin-Eosin (H&E) staining were examined semi-quantitatively under a Leica DM500 microscope and images has been captured.

Histological Evaluation and Maturation of Seminiferous Tubules

To perform light microscopic evaluations, seminiferous tubule diameter and germinal epithelium thickness (25) were measured in 30 randomly selected seminiferous tubules per section at X10 magnification. To assess spermatogenesis in the same seminiferous tubules, a modified Johnsen scoring system was used (26). Spermatogenic cells in the seminiferous tubules examined by using a Leica DM500 microscope were evaluated based on their maturation and density, receiving scores from 1 to 10 according to the following scoring table (Table 1) (26)

Table 1: Modified Johnsen scoring system

10: Complete spermatogenesis with mature sperm cells
9: Few sperm cells present with irregular germinal epithelium
8: Fewer than 10 sperm cells (less than 5-10)
7: No sperm cells, but spermatids are present
6: No sperm cells, fewer than 10 spermatids (less than 5-10)
5: No sperm cells or spermatids, but spermatocytes are present
4: No sperm cells or spermatids, fewer than 5 spermatocytes
3: Only spermatogonia are present as germ cells
2: No germ cells, only Sertoli cells are present
1: No cells in the seminiferous tubule

Immunohistochemically analysis

Determination of CD68 Expression in Testicular Tissue

The immunoregulatory phenotype of testicular macrophages is essential for the preservation of normal testicular function. CD68 staining is used primarily to identify and visualize macrophages and monocytes in tissues (27). Immunohistochemically staining was performed using the avidin-biotin-peroxidase (ABC) complex method with some minor modifications (28, 29). Sections of 7 µm thickness were obtained from the blocked tissues and deparaffinized.

A primary antibody CD68 (Rabbit polyclonal IgG, ab125212, Abcam, London, UK) diluted at 1:200 was used in conjunction with the Thermo Scientific™ TP-015-HA commercial kit. After applying the AEC chromogen, the sections were counterstained with Mayer's hematoxylin and examined under a light microscope. The prepared slides were analysed and captured using a Leica DM500 microscope. Immunoreactivity was scored according to its extent (0.1: <25% cells, 0.4: 26-50%, 0.6: 51-75%, 0.9: 76-100%) and intensity (0: no staining, +0.5: weak, +1: medium, +2: moderate, +3: strong), and a histoscore was calculated by multiplication. The lowest possible histoscore was 0.00 and the highest possible histoscore was 2.70.

Statistical analysis

The results are presented as mean ± SEM. Data were analysed with the SPSS 26.0 (licensed for Adıyaman University, Turkey). Group comparisons were conducted using one-way analysis of variance (ANOVA), followed by the Tukey–HSD test. Statistical significance was considered at $p < 0.05$.

Results

Biochemical results

Serum testosterone levels

The serum testosterone levels were significantly lower in the CCl_4 group (1.73 ng/ml) compared to the control group (3.90 ng/ml, $p < 0.05$). There was no significant difference in testosterone levels between the PJ group (3.87 ng/ml), the CCl_4+PJ group (3.76 ng/ml), and the control group. However, testosterone levels in the CCl_4 group were significantly lower than in the PJ group ($p < 0.05$), and testosterone levels were higher in the CCl_4+PJ group compared to the CCl_4 group ($p < 0.05$, Figure 1.A).

MDA levels

The MDA concentration in the testis was significantly higher in the CCl_4 group (0.30 nmol/g) compared to the control group (0.20 nmol/g, $p < 0.05$). However, there was no significant difference in MDA levels between the PJ group (0.21 nmol/g), the CCl_4+PJ group (0.22 nmol/g), and the control group. Additionally, MDA levels in the PJ and CCl_4+PJ groups were significantly lower than those in the CCl_4 group ($p < 0.05$, Figure 1.B).

GSH levels

The reduced GSH levels were significantly lower in the CCl_4 group (0.062 nmol/mg) compared to the control group (0.081 nmol/mg, $p < 0.05$). There was no significant difference in GSH levels between the PJ group (0.082 nmol/mg), the CCl_4+PJ group (0.074 nmol/mg), and the control group. GSH levels in the PJ group were higher compared to the CCl_4 group. Additionally, there was no significant difference in GSH levels between the CCl_4+PJ group and the PJ group ($p > 0.05$, Figure 1.C).

CaE activity

CaE activity was significantly higher in the PJ group (2566.79 nmol/mg) and the CCl_4+PJ group (1182.90 nmol/

mg) compared to the control group (585.53 nmol/mg, $p < 0.05$). Additionally, CaE activity in the CCl4+PJ group was higher than in the CCl4 group (371.60 nmol/mg, $p < 0.05$). However, there was no significant difference in CaE activity between the CCl4 group and the control group ($p > 0.05$, Figure 1.D).

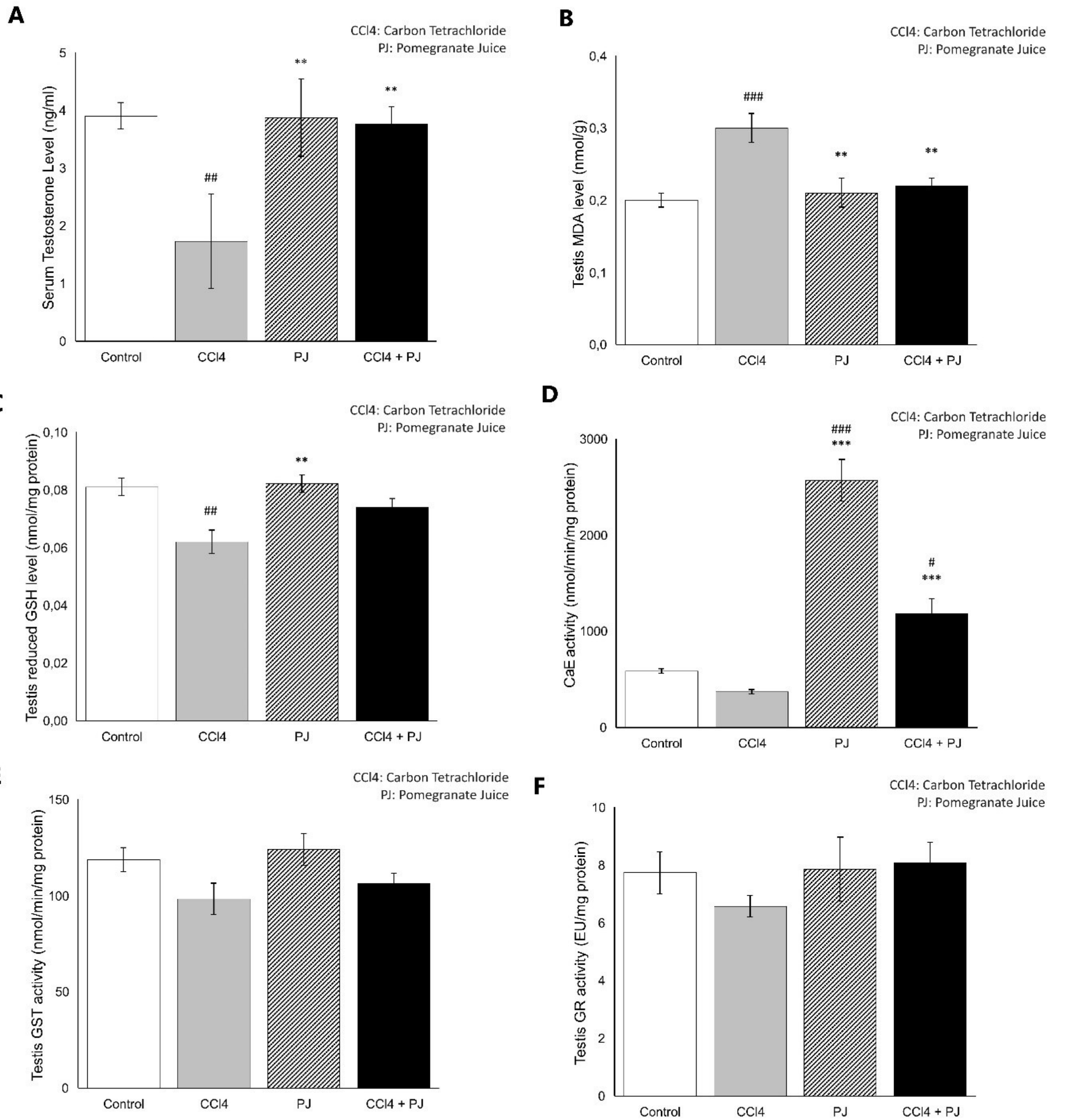

Figure 1: A. Effects of CCl4 and PJ on the serum testosterone levels in male rat. The data represent as mean ± SEM (n=7). #: p<0.05 compared to the control group. *: p<0.05 compared to the CCl4 group. B. Effects of CCl4 and PJ on the MDA level in male rats. The data represent as mean ± SEM (n=7). #: p<0.05 compared to the control group. *: p<0.05 compared to the CCl4 group. C. Effects of CCl4 and PJ on the reduced GSH level in male rats. The data represent as mean ± SEM (n=7). #: p<0.05 compared to the control group, *: p<0.05 compared to the CCl4 group. D. Effects of CCl4 and PJ on the CaE activity in male rats. The data represent as mean ± SEM (n=7). #: p<0.05 compared to the control group, *: p<0.05 compared to the CCl4 group. E. Effects of CCl4 and PJ on the GST activity in male rats. The data represent as mean ± SEM (n=7). #: p<0.05 compared to the control group, *: p<0.05 compared to the CCl4 group. F. Effects of CCl4 and PJ on the GR activity in male rats. The data represent as mean ± SEM (n=7). #: p<0.05 compared to the control group, *: p<0.05 compared to the CCl4 group

GST activity

GST activity did not significantly differ among the CCl4 group (98.2 nmol/mg), PJ group (123.97 nmol/mg), CCl4+PJ group (106.16 nmol/mg), and the control group (118.47 nmol/mg, $p > 0.05$). Additionally, there was no significant difference in GST activity between the CCl4 group and both the PJ and CCl4+PJ groups ($p > 0.05$, Figure 1.E).

GR activity

GR activity did not significantly differ among the CCl4 group (6.57 nmol/mg), PJ group (7.85 nmol/mg), CCl4+PJ group (8.07 nmol/mg), and the control group (7.73 nmol/mg, $p > 0.05$). Additionally, there was no significant difference in GR activity between the CCl4 group and both the PJ and CCl4+PJ groups ($p > 0.05$, Figure 1.F).

Histopathological findings

Haematoxylin and Eosin findings

In the H&E sections of both the control and PJ-treated groups, the testicular tissues exhibited similar histological structures. The seminiferous tubules appeared organized and tightly packed, each surrounded by a basal membrane with myoid cells, and covered by seminiferous epithelium. The seminiferous epithelium consisted of Sertoli cells and layers of germ cells at various stages of development, including spermatogonia, primary and secondary spermatocytes,

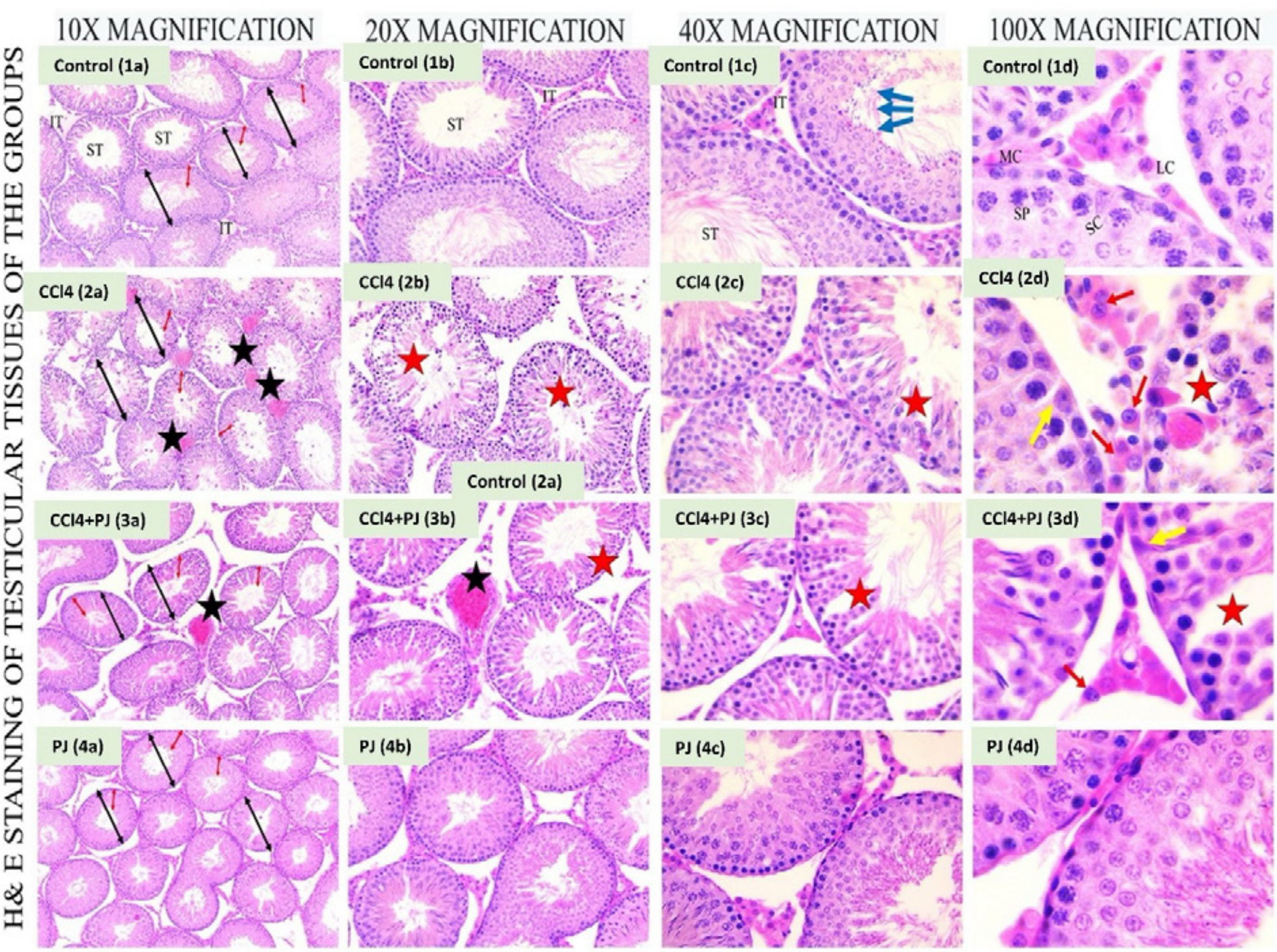

Figure 2: Testicular tissues from all groups were stained with H&E and observed at different magnifications. In the control (1a) and PJ groups (4a), tubule diameter (black double-headed arrow) and germinal epithelium thickness (red double-headed arrow) were similar, with normal seminiferous tubule. Spermatids and spermatozoa (blue arrow) were densely present in the lumen. In contrast, the CCl4 group (2a) showed reduced tubule diameter and decreased germinal epithelium height compared to the control (1a), along with congestion and edema in the interstitial area (black star). Seminiferous tubules exhibited vacuolated germinal epithelium (red star), apoptotic Leydig cells (red arrow), and apoptotic cells among Sertoli cells (yellow arrow). In the CCl4+PJ group (3a), tissue and cell damage were noticeably reduced compared to the CCl4 group (2a). (ST: seminiferous tubule, IT: interstitial tissue, MC: myoid cell, LC: Leydig cell, SP: spermatogenic cells, SC: Sertoli cell)

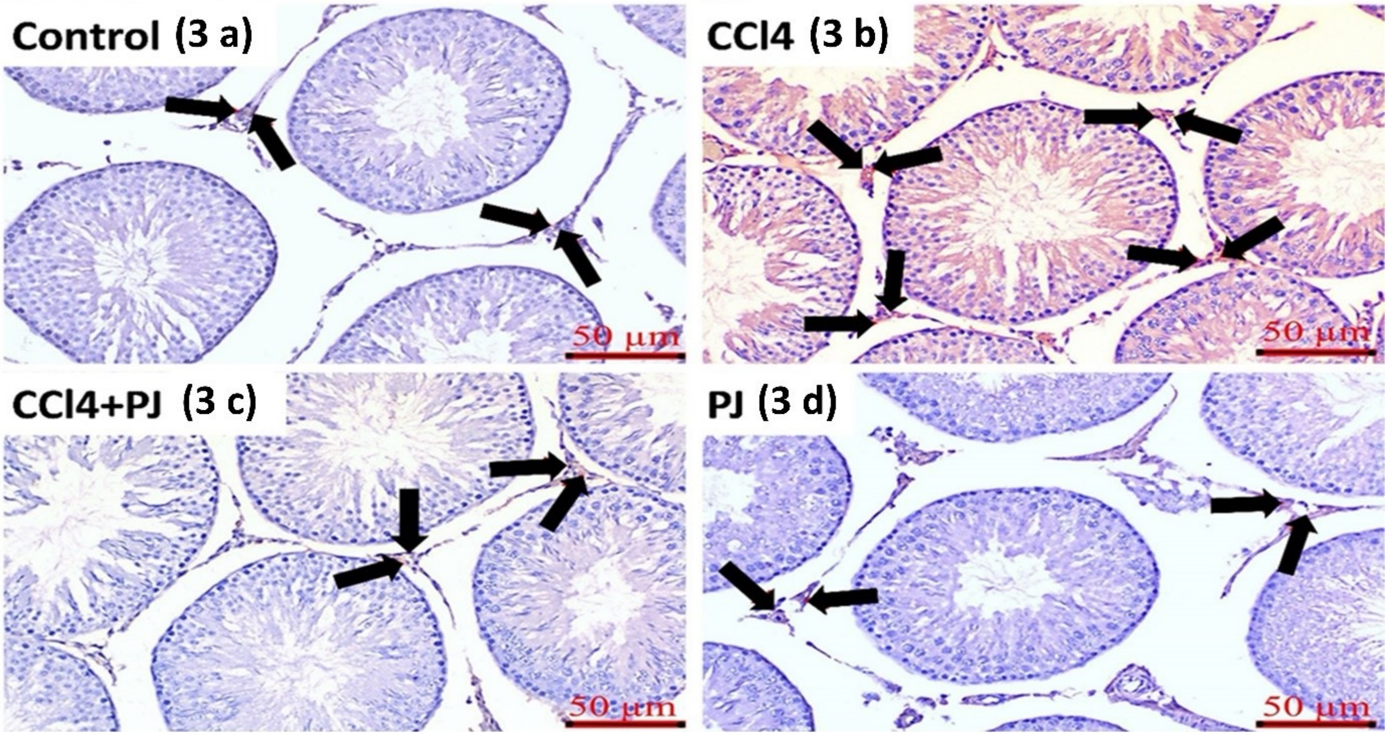

Figure 3: CD68 immunoreactivity (red arrow) was observed in the testicular tissue. Compared to the control group (3a), increased CD68 immunoreactivity was observed in the CCl4 group (3b). However, the CCl4+PJ group (3c) showed decreased CD68 immunoreactivity compared to the CCl4 group (3b). (Immunohistochemical staining, AEC chromogen, scale bar: 50 µm)

spermatids, and spermatozoa. Spermatogonia located on the basal membrane were characterized by dark nuclei, while large primary spermatocytes, with prominent heterochromatic nuclei, were nearby, some showing mitotic figures. Spermatids and spermatozoa were present within the tubules. Sertoli cells appeared as tall, columnar cells extending from the basal membrane to the lumen. Leydig cells, large polygonal cells with acidophilic cytoplasm, appeared normal in the interstitial spaces between the tubules (Figure 2). In the H&E sections of the CCl4 group, compared to the control group, there was evidence of reduced and disrupted seminiferous tubules, with vacuolated germinal epithelium and apoptotic cells located between the basal spermatogonia and Sertoli cells. Detached germ cells were frequently observed in the tubular lumens. Additionally, the cytoplasm of Leydig cells was intensely eosinophilic. Numerous congested blood vessels and vascular congestion were observed, along with apoptotic Leydig cells exhibiting an acidophilic appearance. (Figure 2).

In the H&E sections of the CCl4+PJ-treated group, compared to the CCl4 group, a significant increase in the layers of spermatogenic cells within the seminiferous tubules was observed. However, apart from the presence of dilated numerous congested blood vessels in the interstitial spaces, and the disrupted appearance of some tubules, the histological findings were similar to those of the control and PJ groups (Figure 2).

Findings of seminiferous tubule diameter and germinal epithelium thickness

Seminiferous tubule diameter

Measurements of seminiferous tubule diameter in the testicular tissues of all groups showed similar values between the control (Figure 2a) and PJ (Figure 2d) groups ($p=0.871$). In the CCl4 group (Figure 2b), the seminiferous tubule diameter was significantly decreased compared to the control group ($p<0.05$). However, the CCl4 + PJ group (Figure 2c) showed a statistically significant increase in tubule diameter compared to the CCl4 group (Table 2).

Table 2: Seminiferous tubule diameters (µm)

Groups	Median (mean-max)	<0.05
Control	366, 97 (281, 31-390, 16)	
CCl4	165, 97(117, 03-198, 38) [a]	
CCl4+PJ	261, 31(227, 03 -275, 16) [b]	
PJ	344, 61 (315, 57-384, 93)	

[a]Compared to the CCl4 group, [b]Compared to the CCl4 + PJ group, ($p < 0.05$) (CCl4: Carbon tetrachloride, PJ: Pomegranate juice)

Germinal epithelium thickness

In the testicular tissues of all groups, the germinal epithelium thickness was similar between the Control (Figure 2a) and PJ (Figure 2d) groups (p=0.948). In the CCl4 group (Figure 2b), the germinal epithelium thickness was significantly reduced compared to the Control group 320 (p<0.05). However, the CCl4 + PJ group (Figure 2c) showed a statistically significant increase 321 in germinal epithelium thickness compared to the CCl4 group (Table 3).

Table 3: Germinal epithelium thickness

Groups	Median (mean-max)	<0.05
Control	120, 42 (120, 63-172, 33)	
CCl4	47, 58(21, 55-55, 66) [a]	
CCl4+PJ	115, 27 (100, 26-120, 44) [b]	
PJ	138, 54 (124, 47-218, 23)	

[a]Compared to the CCl4 group, [b]Compared to the CCl4 + PJ group, (p < 0.05) (CCl4: Carbon tetrachloride, PJ: Pomegranate juice)

Johnsen scoring findings

According to the modified Johnsen scoring used to evaluate spermatogenesis in seminiferous tubules, the Control group (Figure 2a) and PJ group (Figure 2d) showed similar results (p=0.845). Spermatogenesis was significantly decreased in the CCl4 group (Figure 2b) compared to the Control group (p<0.05). However, in the CCl4 + PJ group, spermatogenesis 332 was significantly increased compared to the CCl4 group (Figure 2c) (Table 4).

Table 4: Johnsen scoring findings

Groups	Median (mean-max)	<0.05
Control	8, 95 (8, 42-10, 16)	
CCl4	2, 96(2, 64-3, 84) [a]	
CCl4+PJ	7, 32 (5, 21-7, 95) [b]	
PJ	7, 51 (637-8, 34)	

[a]Compared to the CCl4 group, [b] Compared to the CCl4 + PJ group, (p < 0.05) (CCl4: Carbon tetrachloride, PJ: Pomegranate juice)

Immunohistochemical findings

CD68 Immunoreactivity Levels in Testicular Tissue

According to the results of immunohistochemical staining for CD68 analyzed under a light microscope, CD68 immunoreactivity was observed in the interstitial area (red arrow) of testicular tissue. CD68 immunoreactivity was similar between the control group (Figure 3a) and PJ group (Figure 3d) (p=0.816). In the CCl4 group (Figure 3b), CD68 immunoreactivity was significantly increased compared to the control group (p<0.05). However, in the CCl4 + PJ group, it was significantly decreased compared to the CCl4 group (Figure 3c) (Table 5).

Table 5: CD68 Immunoreactivity (Histoscore)

Groups	Median (mean-max)	<0.05
Control	0, 20 (0, 10-0, 30)	
CCl4	1, 80(1, 20-2, 40) [a]	
CCl4+PJ	0, 20(0, 20 -0, 40) [b]	
PJ	0, 20 (0, 10-0, 20)	

[a]Compared to the CCl4 group, [b]Compared to the CCl4 + PJ group, (p < 0.05) (CCl4: Carbon tetrachloride, PJ: Pomegranate juice)

Discussion

Selenium In this study, we investigated the effects of CCl4-induced damage in the testes and the potential protective role of PJ against these effects by evaluating histological and biochemical parameters. CCl4 reduces testosterone levels through a combination of hepatic damage, oxidative stress, inflammatory responses, and direct testicular toxicity, all of which impair the normal production and regulation of testosterone (30). Our findings indicate that CCl4 exposure leads to a decrease in serum testosterone levels. While long-term PJ treatment alone did not affect testosterone levels, it successfully restored the decreased testosterone levels induced by CCl4. CCl4 increased MDA levels in testicular tissue, while long-term PJ treatment reduced elevated MDA levels. CCl4 exposure increased testicular GSH levels, while long-term PJ treatment did not have any effect on GSH levels. While CCl4 exposure alone did not affect CaE activity, we observed that PJ alone significantly increased CaE levels. However, CCl4 exposure reduced the CaE levels increased by PJ. Neither GST nor GR activities were affected by CCl4 exposure or PJ treatment. According to our histopatological analysis, CCl4 notably caused decreased spermatogenesis and vacuole formation in seminiferous tubules and caused interstitial edema. Long-term PJ treatment reduced this histopathological lesions, but the damage caused by CCl4 did not fully recovered. Immunohistochemical staining results showed that increased macrophage activity in animals exposed to CCl4 was reduced with PJ treatment. Despite similar studies (31, 32) evaluating the antioxidant and histopathological effects of PJ in testes, our study adds novelty to the literature by evaluating macrophage activity and introducing new data in oxidative processes related to CCl4 exposure.

Testosterone, the primary male sex hormone produced in the testicles, plays a crucial role in normal male sexual development and function (33). The data of CCl4 reduced serum testosterone levels in our study was consistent with the literature (32). Previous studies on the effects of PJ on serum testosterone levels have shown mixed results: one found no effect (16), while another reported an increase (32). In our study, we also found that PJ alone did not affect serum testosterone levels. These discrepancies may be due to variations in the components of PJ used. However, consistent with other studies (32), we observed that PJ significantly mitigated the CCl4-induced reduction in testosterone, nearly restoring it to control levels. Additionally, an increase in free radicals causes excessive production of MDA, indicating the oxidation of polyunsaturated fatty acids within cells (34). It is known that the plasma membrane of testicular cells contains a high concentration of polyunsaturated fatty acids, it becomes more susceptible to oxidation (34). We found that, consistent with the literature, we found that CCl4 increased testicular MDA levels and that PJ reduced this increase (35). CaE, also found in testis, plays essential roles in the breakdown of various endogenous esters, drugs containing esters, and toxic substances found in the environment (36). GSH is an antioxidant that protects cells from the toxic effects of reactive oxygen species such as free radicals, peroxides, heavy metals and chemical toxins (37). Previous studies have reported that CCl4 increases GSH levels in both renal (38) and testicular tissue (32). However, our data indicate a reduction in GSH levels following CCl4 exposure. Literature suggests that GSH levels may vary with age (39). The observed decrease in GSH levels in our study could be attributed to age-related changes. There are some studies suggest that alterations in the testicular antioxidant defence profile in rats are influenced by age (40, 41). Contrary to the literature (32), neither CCl4 nor PJ showed any effect on GST and GR activities in testicular tissue in our study. This difference, as previously mentioned, could be the result of the age-related decreases in cellular glutathione-related antioxidant capacity. However, we found that the chemical toxin CCl4 had no effect on CaE activity. A previous study demonstrated that CCl4 reduces liver CaE activity (42). The unaffected testicular CaE activity according to our data led us to conclude that expression levels, isoforms, and specific roles of CaE can vary significantly between tissues such as the testis and the brain (43) may so CaEs may exhibit different responses to various chemical toxins. Interestingly again, we found that PJ alone increased CaE activity, while when given together with CCl4, this activity decreased. Despite the decrease, the activity in the CCl4 + PJ group was still higher than in the control group. These data regarding CaE activity are the novel findings of our study. However, further research is needed to investigate this different CaE activity. The histopathological findings obtained in our study were consistent with the literature (44).

Conclusion

IIn conclusion, our study showed that CCl4 increases MDA levels in testicular tissue, leading to oxidative stress and disruption of cellular antioxidant systems. Pomegranate juice mitigated these effects using alternative antioxidant mechanisms rather than glutathione. Pomegranate juice alone also enhanced CaE activity, which is reduced by CCl4 exposure. These data are novel findings of our study. Furthermore, pomegranate juice restored serum testosterone levels and promoted recovery in testicular histopathology

Acknowledgements

The chemical composition of the pomegranate juice (PJ) was examined within the framework of the project "My City is Adıyaman, " identified by project number: TRC1/18/KBG/0.036. Financial support for the project was provided through the Development of Urban Culture and Urban Awareness Financial Support Program.

Declaration of interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Author Contributions Conception and design: Ömer Faruk Kalkan, Zafer Şahin, Ahmet Özkaya. Acquisition of data and analysis: Ahmet TÜRK, Cihan Çitil, Miraç Uçkun, Ertan Yoloğu, Yusuf Özay, Şeyda Çağri Bülbül, Nadir Bilgin Akgül, Akın Kirbaş. Revision for important intellectual content: Ömer Faruk Kalkan and Zafer Şahin. Supervision: Ömer Faruk Kalkan and Zafer Şahin.

Ethics approval. The ethical treatment of the rats followed approved guidelines for the use of laboratory animals and complied with the regulations of the Adıyaman University Animal Experiments Centre Ethics Committee, Türkiye (protocol number: 2022/17).

Conflict of Interest. Authors declare no conflict of interest.

Data Availability Statement. Our manuscript has no associated datat.

References

1. Agarwal A, Mulgund A, Hamada A, Chyatte MR. A unique view on male infertility around the globe. Reprod Biol Endocrinol 2015; 13: 37. doi: 10.1186/s12958-015-0032-1

2. Babakhanzadeh E, Nazari M, Ghasemifar S, Khodadadian A. Some of the factors involved in male infertility: a prospective review. Int J Gen Med 2020; 13: 29–41. doi: 10.2147/IJGM.S241099

3. Rodprasert W, Toppari J, Virtanen HE. Environmental toxicants and male fertility. Best Pract Res Clin Obstet Gynaecol 2023; 86: 102298. doi: 10.1016/j.bpobgyn.2022.102298

4. Louria DB, Bogden JD, Reynolds ES. The dangers from limited exposure to carbon tetrachloride. CRC Crit Rev Toxicol 1980; 7(2): 177–88. doi: 10.3109/10408448009037488

5. Ruprah M, Mant TGK, Flanagan RJ. Acute carbon tetrachloride poisoning in 19 patients: implications for diagnosis and treatment. Lancet 1985; 325(8436): P1027–9.

6. Weber LWD, Boll M, Stampfl A. Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. Crit Rev Toxicol 2003; 33(2): 105–36. doi: 10.1080/713611034

7. Rahal A, Kumar A, Singh V, et al. Oxidative stress, prooxidants, and antioxidants: the interplay. BioMed Res Int 2014; 2014: 761264. doi: 10.1155/2014/761264

8. Sönmez M, Türk G, Çeribaşı S, et al. Quercetin attenuates carbon tetrachloride-induced testicular damage in rats. Andrologia 2014; 46(8): 848–58. doi: 10.1111/and.12159

9. Sahreen S, Khan MR, Khan RA. Ameliorating effect of various fractions of rumex hastatus roots against hepato- and testicular toxicity caused by CCl4. Oxid Med Cell Longev 2013; 2013: 325406. doi: 10.1155/2013/325406

10. Perron NR, Brumaghim JL. A Review of the antioxidant mechanisms of polyphenol compounds related to iron binding. Cell Biochem Biophys 2009; 53(2): 75–100. doi: 10.1007/s12013-009-9043-x

11. Zehiroglu C, Ozturk Sarikaya SB. The importance of antioxidants and place in today's scientific and technological studies. J Food Sci Technol 2019; 56(11): 4757–74. doi: 10.1007/s13197-019-03952-x

12. Sharma A, Shrivastava D. Psychological problems related to infertility. Cureus 2022; 14(10): e30320. doi: 10.7759/cureus.30320

13. Li X, Zhang B, Yang H, Zhang L, Lou H, Zheng S. The emergence of natural products as potential therapeutics for male infertility. Andrology 2024; 12(6): 1191–208. doi: 10.1111/andr.13588

14. Gil MI, Tomás-Barberán FA, Hess-Pierce B, Holcroft DM, Kader AA. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. J Agric Food Chem 2000; 48(10): 4581–9. doi: 10.1021/jf000404a

15. Kiliçgün H, Arda N, Uçar EÖ. Identification of longevity, fertility and growth-promoting properties of pomegranate in Caenorhabditis elegans. Pharmacogn Mag 2015; 11(42): 356–9. doi: 10.4103/0973-1296.153089

16. Türk G, Sönmez M, Aydin M, et al. Effects of pomegranate juice consumption on sperm quality, spermatogenic cell density, antioxidant activity and testosterone level in male rats. Clin Nutr 2008; 27(2): 289–96. doi: 10.1016/j.clnu.2007.12.006

17. Lee HY, Lee GH, Yoon Y, Chae HJ. R. verniciflua and E. ulmoides extract (ILF-RE) protects against chronic CCl4-induced liver damage by enhancing antioxidation. Nutrients 2019; 11(2): 382. doi: 10.3390/nu11020382

18. Çiftçi H, Er Çalışkan Ç, Kar F, Özkaya A. Evaluation of the effects of pomegranate juice on hepato-nephrotoxicity in male rats exposed to aluminum. KSÜ Tarım Doğa Derg 2022; 25(2): 215–22. doi: 10.18016/ksutarimdoga.vi.896611

19. Placer ZA, Cushman LL, Johnson BC. Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems. Anal Biochem 1966; 16(2): 359–64. doi: 10.1016/0003-2697(66)90167-9

20. Moron M, Depierre J, Mannervik B. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochim Biophys Acta 1979; 582(1): 67–78. doi: 10.1016/0304-4165(79)90289-7

21. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248–54. doi: 10.1016/0003-2697(76)90527-3

22. Carlberg I, Mannervik B. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J Biol Chem 1975; 250(14): 5475–80.

23. Nousiainen U, Törrönen R. Differentiation of microsomal and cytosolic carboxylesterases in the rat liver by in vivo and in vitro inhibition. Gen Pharmacol 1984; 15(3): 223–7. doi: 10.1016/0306-3623(84)90163-0

24. Santhoshkumar P, Shivanandappa T. In vitro sequestration of two organophosphorus homologs by the rat liver. Chem Biol Interact 1999; 119/120: 277–82. doi: 10.1016/s0009-2797(99)00037-x

25. Bal R, Türk G, Tuzcu M, et al. Protective effects of nanostructures of hydrated C60 fullerene on reproductive function in streptozotocin-diabetic male rats. Toxicology 2011; 282(3): 69–81. doi: 10.1016/j.tox.2010.12.003

26. Oliveira Filho AB, Souza RS, Azeredo-Oliveira MTV, Peruquetti RL, Cedenho AP. Microdissection testicular sperm extraction causes spermatogenic alterations in the contralateral testis. Gen Mol Res 2010; 9(3): 1405–13. doi: 10.4238/vol9-3gmr860

27. Manduch M, Dexter DF, Jalink DW, Vanner SJ, Hurlbut DJ. Undifferentiated pancreatic carcinoma with osteoclast-like giant cells: report of a case with osteochondroid differentiation. Pathol Res Pract 2009; 205(5): 353–9. doi: 10.1016/j.prp.2008.11.006

28. Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 1981; 29(4): 577–80. doi: 10.1177/29.4.6166661

29. Metin TO, Turk A, Yalcin A. Beta-glucan: a powerful antioxidant to overcome cyclophosphamide-induced cardiotoxicity in rats. Med Sci 2022; 1(4): 1431–5. doi: 10.5455/medscience.2022.06.143

30. Unsal V, Cicek M, Sabancilar İ. Toxicity of carbon tetrachloride, free radicals and role of antioxidants. Rev Environ Health 2020; 36(2): 279–95. doi: 10.1515/reveh-2020-0048

31. Annaç E, Uçkun M, Özkaya A, et al. The protective effects of pomegranate juice on lead acetate-induced neurotoxicity in the male rat: a histomorphometric and biochemical study. J Food Biochem 2022; 46(4): e13881. doi: 10.1111/jfbc.13881

32. Al-Olayan EM, El-Khadragy MF, Metwally DM, Moneim AEA. Protective effects of pomegranate (Punica granatum) juice on testes against carbon tetrachloride intoxication in rats. BMC Complement Altern Med 2014; 14: 164. doi: 10.1186/1472-6882-14-164

33. Ide H. The impact of testosterone in men's health. Endocr J 2023; 70(7): 655–62. doi: 10.1507/endocrj.EJ22-0604

34. Sahin Z, Ozkaya A, Cuce G, Uckun M, Yologlu E. Investigation of the effect of naringenin on oxidative stress-related alterations in testis of hydrogen peroxide-administered rats. J Biochem Mol Toxicol 2017; 31(9): e21928. doi: 10.1002/jbt.21928

35. Türk G, Çeribaşı S, Sönmez M, et al. Ameliorating effect of pomegranate juice consumption on carbon tetrachloride-induced sperm damages, lipid peroxidation, and testicular apoptosis. Toxicol Ind Health 2016; 32(1): 126–37. doi: 10.1177/0748233713499600

36. Wang D, Zou L, Jin Q, Hou J, Ge G, Yang L. Human carboxylesterases: a comprehensive review. Acta Pharm Sin B 2018; 8(5): 699–712. doi: 10.1016/j.apsb.2018.05.005

37. Zhang W, Gao J, Lu L, et al. Intracellular GSH/GST antioxidants system change as an earlier biomarker for toxicity evaluation of iron oxide nanoparticles. NanoImpact 2021; 23: 100338. doi: 10.1016/j.impact.2021.100338

38. Eassawy MMT, Ismail AFM. Protective effect of chicory and/or artichoke leaves extracts on carbon tetrachloride and gamma-irradiation-induced chronic nephrotoxicity in rats. Environ Toxicol 2024; 39(3): 1666–81. doi: 10.1002/tox.24060

39. Zhu Y, Carvey PM, Ling Z. Age-related changes in glutathione and glutathione-related enzymes in rat brain. Brain Res 2006; 1090(1): 35–44. doi: 10.1016/j.brainres.2006.03.063

40. Samanta L, Sahoo A, Chainy GBN. Age-related changes in rat testicular oxidative stress parameters by hexachlorocyclohexane. Arch Toxicol 1999; 73: 96–107. doi: 10.1007/s002040050593

41. Salomon TB, Hackenhaar FS, Almeida AC, et al. Oxidative stress in testis of animals during aging with and without reproductive activity. Exp Gerontol 2013; 48(9): 940–6. doi: 10.1016/j.exger.2013.06.010

42. Wei Y, Ma D, Fan Y, et al. Environmental carbon tetrachloride exposure disrupts the liver structure and metabolic detoxification function in mice via p38MAPK/NF-κB/NLRP3 pathway. Food Agric Immunol 2022; 33(1): 235–51. doi: 10.1080/09540105.2022.2060192

43. Hosokawa M. Structure and catalytic properties of carboxylesterase isozymes involved in metabolic activation of prodrugs. Molecules 2008; 13(2): 412–31. doi: 10.3390/molecules13020412

44. Minisy FM, Shawki HH, El Omri A, et al. Pomegranate seeds extract possesses a protective effect against tramadol-induced testicular toxicity in experimental rats. BioMed Research International 2020; 2020: 2732958. doi: 10.1155/2020/2732958

Zdravljenje s sokom granatnega jabolka preprečuje poškodbe mod zaradi ogljikovega tetraklorida (CCl4) pri podganah: biokemična in histološka študija

Ö. F. Kalkan, A. Türk, C. Çitil, M. Uçkun, A. Özkaya, E. Yoloğlu, Y. Özay, Ş. Ç. Bülbül, N. B. Akgül, Z. Şahin, A. Kirbaş

Izvleček: Raziskali smo neželene učinke ogljikovega tetraklorida (CCl4) na tkivo mod in preučili zaščitne učinke soka granatnega jabolka (PJ) ob izpostavljenosti CCl4. 28 samcev podgan wistar albino je bilo naključno razdeljenih v štiri skupine: kontrolno, PJ, CCl4 in CCl4 + PJ. Merili smo raven testosterona v serumu in ocenili parametre karboksilesteraze (CaE), malondialdehida (MDA), glutationa (GSH), glutation reduktaze (GR) in glutation S-transferaze (GST). Histopatološke spremembe so bile pregledane z uporabo imunoreaktivnosti CD68 za ugotavljanje aktivnosti makrofagov. Analiza je pokazala znatno povečanje aktivnosti CaE v skupini PJ v primerjavi s kontrolno skupino ($p < 0{,}05$). Izpostavljenost CCl4 je zmanjšala aktivnost CaE, v skupini CCl4 + PJ pa se je aktivnost CaE delno izboljšala s PJ ($p < 0{,}05$). PJ je tudi značilno znižal povišano raven MDA, ki jo je povzročil CCl4 ($p < 0{,}05$). CCl4 je zmanjšal raven reduciranega glutationa (GSH) ($p < 0{,}05$), vendar PJ ni vplival na raven GSH ($p > 0{,}05$). Niti CCl4 niti PJ nista vplivala na aktivnost glutation reduktaze (GR) in glutation S-transferaze (GST) ($p > 0{,}05$). PJ je torej odpravil histološke poškodbe in obnovil znižano raven testosterona. Poleg tega je ublažil oksidativni stres, ki ga je povzročil CCl4, tako da je namesto glutationa uporabil alternativni antioksidativni sistem.

Ključne besede: toksičnost CCl4, neplodnost, sok granatnega jabolka, karboksilestraza, oksidativni stres, histopatologija