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FULL PAPER

Toxicology

Pomegranate peel extract, N-Acetylcysteine and their combination with Ornipural alleviate Cadmium-induced toxicity in rats

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ABSTRACT. Cadmium is a major environmental pollutant and a highly toxic metal. It was aimed to determine the effects of pomegranate peel extract (PPE), N-acetylcysteine (NAC) alone and along with Ornipural on cadmium-induced toxicity. Forty-six Wistar Albino male rats were divided into 6 groups and the groups were formed into healthy control, Cadmium group (5 mg/kg/day, oral), Cadmium + Pomegranate peel extract (500 mg/kg, oral), Cadmium + N-acetylcysteine (100 mg/kg, oral), Cadmium + Pomegranate peel extract (500 mg/kg, oral) + Ornipural (1 mL/kg, subcutaneous) and Cadmium + N-acetylcysteine (100 mg/kg, oral) + Ornipural (1 mL/kg, subcutaneous). Cadmium accumulated heavily in both liver and kidney tissue. The administration of N-acetylcysteine and pomegranate peel extract alone reduced cadmium levels in both tissues. N-acetylcysteine treatment prevented the increase in ALT and MDA levels by cadmium damage. N-acetylcysteine + Ornipural treatment inhibited the increase in liver 8-OHdG level in the liver. N-acetylcysteine and N-acetylcysteine + Ornipural treatments prevented the reduced serum MMP2 level. N-acetylcysteine and Pomegranate peel extract + Ornipural treatments significantly reduced the increased liver iNOS level in the liver. In conclusion, NAC therapy may be a successful treatment option for cadmium toxicity. However, further research is needed on the effects of PPE and Ornipural combinations for the treatment of cadmium toxicity. In future studies, various doses of these treatment options (with chelators) should be investigated for cadmium toxicity.

KEYWORDS: cadmium, heavy metal, n-acetylcysteine, pomegranate, toxicity

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Cadmium (Cd) is a heavy metal that is toxic to humans and animals. Cadmium is generally used as a stabilizer in polyvinyl chloride products, color pigments, Ni-Cd batteries, and in industry [29]. Cadmium is present in a significant amount in the environment due to human activities [52]. Cadmium pollution in the environment has increased with the increase of industrial waste. Cadmium exposure to humans and animals has increased through water, air, and soil contamination [29]. Cadmium accumulation in the kidneys, liver, lungs, brain, testes, heart, and central nervous system causes adverse effects [54]. Humans may be exposed to cadmium toxicity by the oral and inhalation route. They are exposed to cadmium toxicity by eating shellfish such as mussels and oysters, and by consuming foods grown in soils where waste batteries pollute the environment [29, 64]. In particular, cadmium toxicity can occur through smoking by inhalation [7]. One of the main routes of chronic Cd exposure are the consumption of rice grown in Cd-contaminated waters by people in Toyama Prefecture (Japan) in 1912. The exposure as known as Itai-Itai disease in Japan, can cause serious problems in the skeletal system associated with osteoporosis [29, 52]. Cattle are among the most susceptible species for cadmium toxicity in the veterinary perspective [3].

Calves exposed to Cd toxicity experience rough coat hair, skin rash, hair loss in various parts of the body, and extreme weakening [44]. It has been reported that the total protein level decreased with liver toxicity in bulls with Cd toxicity [45]. In addition, Cd causes decreased reproductive performance, premature birth, or perinatal death at low concentrations. It also causes growth retardation, decreased milk production, and abortions at high concentrations in cattle and sheep. Cadmium cause liver degeneration in the early stage [44].

The consumption of meat or organs such as the liver and kidneys of aged cattle or sheep is a key factor in Cd toxicity in humans [14]. Cadmium exposure causes various health problems in many organs such as nephrotoxicity, hepatotoxicity, testicular damage,

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pulmonary edema, osteomalacia, non-hypertrophic emphysema, eosinophilia, anemia and damage to the hematopoietic system [29, 69]. In addition, Cd has been classified as a carcinogen by the International Agency for Research on Cancer [9, 68]. Its exceptionally high toxicity is due to its long biological half-life [29].

Although different mechanisms play role in Cd toxicity, Cd mainly induces oxidative stress in the cells. Cadmium affects many cellular activities, such as DNA synthesis, apoptosis, cell proliferation, and differentiation [54]. Cadmium leads to malignancy by activating the c-jun N-terminal kinase (JNK) pathway, increasing the expression of genes involved in cell growth and division. Cd changes the transcription of metal-regulatory transcription factor and nuclear factor-κB (NF-κB), and it triggers tumor development [19]. In addition Cd stimulates carcinogenesis by modulating the intracellular calcium level and reactive oxygen species; this triggers DNA damage and stimulates carcinogenesis [19, 29]. Programmed cell death increases when hepatic cells are exposed to Cd [71]. Furthermore, Cd impairs ATP synthesis and cellular functions in the mitochondrial membrane by increasing the formation of free radicals, consuming cellular glutathione (GSH), and inhibiting the antioxidant system, causing oxidative damage to DNA, lipids and proteins [19, 29].

The most widely used method to reduce Cd toxicity is to inactivate it by binding with chelators (such as curry leaves, or tomatoes). Nevertheless, some chelators have unwanted side effects. The efficacy of chelators is variable in heavy metal toxicity, so chelators alone are not sufficient. New therapeutic agents are needed to reduce Cd toxicity. This necessity has led to the evaluation of natural antioxidants and dietary components for Cd toxicity reduction [75].

Pomegranate peel (*Punica granatum L.*) is rich in flavonoids, tannins, anthocyanins, phenolic and organic acids and they have positive effects in many diseases. Tannins are composed of the ellagitannin and gallotannin families, which can be hydrolyzed to ellagic and gallic acids. The majority of the antioxidant effect of pomegranate is linked to punicalagin, which is from the ellagitannin family. The flavonoid anthocyanin contributes to the improvement of various diseases by antioxidant, anti-inflammatory, and antiproliferative properties. Pomegranate extract has prebiotic, antimicrobial, antidiabetic, and anticancer effects; it is also used in the treatment of neurodegenerative and liver disorders [38]. It has been reported that pomegranate peel increases antioxidant capacity and reduces liver and kidney damage in cadmium-induced toxicity in mice [21, 59].

N-acetylcysteine (NAC) is a cysteine derivative that suppresses oxidative stress and can bind heavy metal ions; it is the precursor to antioxidant GSH [50]. Currently, it is used as an antidote to poisoning from 40 drugs, categorized by the World Health Organization [60]. N-acetylcysteine chelates metal ions such as Cd, mercury, and lead and facilitates their excretion from the body. N-acetylcysteine exhibits an anti-inflammatory effect via the modulation of proinflammatory cytokine synthesis and the inhibition of NF-κB. It also reduces oxidative stress by detoxifying free radicals [50]. In a study on Cd-induced toxicity in rats, NAC reduced the toxic effects of Cd in the kidney without any decrease in Cd in the tissue [39].

Ornipural® (Orn) is a veterinary commercial product containing betaine (15 mg), L-arginine (33.3 mg), L-ornithine (11.8 mg), L-citruline (10 mg) and sorbitol (200 mg). It is approved for the treatment of various diseases/toxications, such as poisoning and hepatonephritis. It has protective properties for the liver and diuretic properties [5].

Betaine is an important component of various foods such as wheat, shellfish, spinach, and sugar beets. It is also found in microorganisms, plants, and animals. Betaine participates in the methionine cycle in the liver and kidneys of humans [11]. Previous literature indicates that betaine is anti-inflammatory [66], anti-apoptotic, and autophagic [70]. In cadmium toxicity, it has been reported to have antioxidant and antiapoptotic effects [31]. L-arginine, one of the essential amino acids, is the precursor to both proteins and urea polyamine, glutamate, creatine, and agmatine. L-arginine is a necessary substrate for the production of nitric oxide (NO), which aids in important role in various physiological processes such as neurotransmission, cytotoxicity and immunity [28]. Although it is defined that L-arginine may be protective against Cd toxicity due to its antioxidant properties [63], its effects on Cd toxicity are not well understood.

Cadmium toxicity is a type of poisoning that is important for both veterinary medicine and human medicine. Left untreated, it can lead to organ failure, poor quality of life and death. For these reasons, in addition to the use of chelator substances for an effective treatment in cases of Cd poisoning, it is necessary to use substances that minimize organ damage.

The present study was hypothesized to determine and compare the effects of the combination of pomegranate peel extract (PPE) and NAC with Ornipural supplementation in an experimental Cd toxicity model in rats. The aim was to determine the effects of PPE alone (500 mg/kg), NAC alone (100 mg/kg) and combinations of these two substances with Ornipural (1 mL/kg) on some organ damage, oxidative status, inflammation, and hematological parameters in rats with experimental Cd toxicity.

MATERIALS AND METHODS

Chemicals

N-acetylcysteine was purchased from Pharmactive Co. (Mukozero 600 mg, effervescent tablet, Istanbul, Turkey), Ornipural was purchased from Vetoquinol S.A. (Ornipural Solution, Lure-Cudex, France), cadmium was purchased from Carlo Erba Reagents S.A.S. (Emmendingen, Germany) and thiopental sodium was purchased from Ulagay (Pental Sodyum, İ.E. Ulagay, İstanbul, Turkey) for this experiment. Total antioxidant capacity (TAC) ELISA kit (Rat total antioxidant status, Cat No: E1710Ra), matrix metalloproteinase 2 and 9 (MMP2 and MMP9) ELISA kit (Rat Matrix Metalloproteinase 2, Cat No: E0315Ra and Rat Matrix Metalloproteinase 9, Cat No: E0321Ra), tumor necrosis factor (TNF)-α ELISA kit (Rat tumor necrosis factor alpha, Cat No: E0764Ra), 8-hydroxy-deoxyguanosine (8-OHdG) ELISA kit (Rat 8-hydroxy-deoxyguanosine, Cat No: E0031Ra), and inducible nitric oxide synthase (iNOS) ELISA kit (Rat inducible nitric oxide synthase, Cat No: E0740Ra) was purchased from Bioassay Technology Laboratory (Shanghai, China).

Animal material

Forty-six Wistar Albino breed 8–12 weeks old (~250 g) male rats (Selçuk University Experimental Medicine Research and Application Center, Konya) were used. The research protocol was reviewed and approved by the Ethics Committee of the Experimental Medical Practice and Research Center of Animal Experiments at Selcuk University (Ethics Committee Decision No: 2019/35). Before the rats were included in the study, health controls were established and implemented. Food and water needs were provided *ad libitum* and the animals were fed with standard rat food. The temperature, light, and humidity levels of the environment of the animals were monitored and controlled. (12/12 hr light/dark-07:30/19:30, 22 ± 2°C, 55 ± 5% humidity).

Pomegranate peel extraction

The extraction of pomegranate peels in the study was carried out according to the method specified by Demir *et al*. [16]. In this method, after the pomegranate peels were treated with 33% ethanol, at 78°C and for 113 min, they were extracted by mixing at the ratio a 200:20 ratio (mL solvent/g plant). The extract was centrifuged at 5,000 rpm for 10 min, then filtered through filter paper at room temperature. The filtrate was concentrated in the evaporator (Heidolph, Laborata 4,000, Schwabach, Germany) and made ready for use.

The phenolic content in the pomegranate peel extract was analyzed according to the method utilized by Demir *et al.* [16]; Gallic acid, Caffeic acid, Ferulic acid, Catechin, 2,4 hydroxybenzoic acid, Chlorogenic acid, Elagic acid, Vanillic acid, Quercetin, Epicatechin, Kaempeferol, Rosmarinic acid, Naringenin, and Punigalagin.

Experimental design

A total of 46 rats were randomly grouped as follows.

Group 1 [Healthy (control, n:6)]: The animals received physiological saline (1 mL/rat/day, orally) simultaneously with the other groups.

Group 2 [Cd (5 mg/kg) (n:8)]: The animals received $CdCl_2$ solution (in 0.5 ml sterile physiological) was administered by oral gavage at a dose of 5 mg/kg/day for 8 weeks [56, 57].

Group 3 [Cd (5 mg/kg) + PPE (500 mg/kg) (n:8)]: PPE (dose of 500 mg/kg/day) was administered as an aqueous solution by intragastric gavage. $CdCl_2$ solution was administered by oral gavage at a dose of 5 mg/kg/day, 30 min after PPE is given for 8 weeks [1, 18].

Group 4 [Cd (5 mg/kg) + PPE (500 mg/kg) + Orn (1 mL/kg) (n:8)]: PPE was administered orally at a dose of 500 mg/kg/day, with a combination of Orn at a dose of 1 mL/kg (subcutanoeusly). $CdCl_2$ solution was administered by oral gavage at a dose of 5 mg/kg/day 30 min after PPE is given for 8 weeks.

Group 5 [Cd (5 mg/kg) + NAC (100 mg/kg) (n:8)]: NAC was orally administered at a dose of 100 mg/kg/day. $CdCl_2$ solution was by oral gavage administered at a dose of 5 mg/kg/day, 30 min after NAC is given for 8 weeks [24].

Group 6 [Cd (5 mg/kg) + NAC (100 mg/kg) + Orn (1 mL/kg) (n:8)]: NAC was administered orally at a dose of 100 mg/kg/day, with a combination of Orn at a dose of 1 mL/kg (subcutanoeusly). $CdCl_2$ solution was administered by oral gavage at a dose of 5 mg/kg/day 30 min after NAC is given for 8 weeks.

Hematological, biochemical, and oxidative status analysis

Following the eight-week experimental period, the blood samples were collected from hearts of the animals under thiopental sodium anesthesia and euthanasia was performed by decapitation. The liver and kidney tissues of the animals were removed and stored at −80°C until analysis. The hematology parameters (WBC, RBC, hemoglobin (Hb), lymphocyte, monocytes, hematocrit) of the blood samples were measured with a hematology analyzer (Mindray Bio-Medical Electronics, Shenzhen, China). The blood samples were added to into serum and anticoagulant (K_3 EDTA) tubes and then they were centrifuged at 4,000 rpm and separated into serum and plasma. The biochemical parameters [Albumin (ALB), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST)] in the serums were analyzed with an autoanalyzer (Abbott, ci8,200, Jiangsu, China). Total antioxidant capacity, MMP2 and MMP9 levels in the serum/plasma samples, and TNF-α, 8-OHdG and iNOS levels in the liver samples were measured by the ELISA reader (MWGt Lambda Scan 200, Winooski, VT, USA) in accordance with the methods of commercial ELISA kit (Bioassay Technology Laboratory, Shanghai, China). In addition, malondialdehyde (MDA) levels in the liver samples were analyzed in a spectrophotometer device (Perkin Elmer, Lambda 25 UV/Vis spectrophometer, Shelton, CT, USA) according to the method of Ohkawa, Ohishi [49].

Cadmium level analysis

The cadmium analysis was performed according to the method specified in Kacar *et al*. [35]. The liver and kidney tissues from each animal were weighed (0.2 g). The tissues were placed in an H_2O_2-HNO_3 (2 mL H_2O_2-6 mL HNO_3) acid mixture and heated in a microwave oven (Milestone Ethos Easy Advanced Microwave Digestion System model, Italy) at 100% power. Then the samples were diluted to a total volume of 20 mL and the amount of Cd in the samples was determined by Atomic Absorption Spectrophotometer (Shimadzu AA-7000, Kyoto, Japan).

Statistical analysis

The data obtained from the study were evaluated with the SPSS 25.0 (SPSS, Inc., Chicago, IL, USA) program. Since the data did not show normal distribution, they were evaluated with the non-parametric Kruskal-Wallis and *post hoc* Dunn-Bonferoni tests. However, since the measurement data were parametric, they were presented as mean ± SEM in the tables. A value of $P<0.05$ was considered statistically significant.

RESULTS

The effects of PPE, NAC, and Orn treatments on some biomarkers in liver tissue and serum in rats with experimental Cd toxicity are shown in Tables 1 and 2, respectively.

Cadmium increased the statistically significant 8-OHdG level in the liver ($P<0.05$). The 8-OHdG levels in the liver were statistically decreased with Cd + NAC + Orn administration from those of Cd group ($P<0.05$). While the liver iNOS level increased from that of Cd group, the increase was improved by NAC and PPE + Orn treatments ($P<0.05$). The liver MDA level decreased from that of the Cd group due to only NAC and NAC + Orn treatments ($P<0.05$).

Cadmium application caused a statistically significant decrease in TAC level while NAC treatment improved the decreased TAC level ($P<0.05$). The serum MMP2 level statistically decreased in the Cd group from that of the control group ($P<0.05$), and it was inhibited by only NAC and NAC + Orn treatments ($P<0.05$).

No statistically significant was observed between the groups in liver TNF-α and serum MMP-9, AST, ALB values ($P>0.05$).

The effects of PPE, NAC, and Orn treatments on some biochemical parameters in rats with experimental Cd toxicity are shown in Table 3. While Cd administration caused a statistically significant increase in serum ALT level, the increase was improved with NAC treatments alone ($P<0.05$).

Cadmium levels in the kidney and liver of the rats are shown in Table 4. At the end of this period, a greater amount of Cd accumulation was observed in the liver and kidney compared to the control group ($P<0.05$). It was determined NAC treatment decreased Cd levels in the liver and kidney, whereas PPE treatment decreased Cd levels only in the liver ($P<0.05$).

The effects of PPE, NAC, and Orn treatments on hematological parameters in rats with experimental Cd toxicity are shown in Table 5. Cadmium caused a statistically significant decrease in hemoglobin level in all groups except Cd + PPE + Orn compared to the control group ($P<0.05$).

DISCUSSION

As a heavy toxic metal that adversely affects human and animal health, Cd causes structural and functional damage to the liver tissue depending on the dosage and exposure duration [20, 21].

Table 1. The effect of pomegranate peel extract (PPE) (mg/kg) and N-acetylcysteine (NAC) (100 mg/kg) alone and in combination with Ornipural (1 mL/kg) treatments on some biomarkers in liver tissue toxicity of Cd (mg/kg) in rats (Mean ± SEM)

Parameter	Control	Cd	Cd + PPE	Cd + PPE + Orn	Cd + NAC	Cd + NAC + Orn
TNF-α (ng/mL)	1,784.85 ± 30.38	2,031.12 ± 135.54	661.65 ± 276.0	1,801.14 ± 80.14	1,850.82 ± 103.70	1,933.26 ± 122.73
8-OHdG (ng/mL)	1.15 ± 0.38	12.08 ± 1.26*	14.72 ± 0.57*	7.18 ± 0.75	9.01 ± 1.33*	2.50 ± 1.00#
İNOS (ng/mL)	39.89 ± 33.07	152.13 ± 20.62*	141.78 ± 36.57*	8.05 ± 1.55#	5.81 ± 1.15#	74.40 ± 38.67
MDA (nmol/mL)	21.70 ± 2.59	24.71 ± 0.61	23.97 ± 1.68	22.74 ± 1.09	17.26 ± 1.56#	17.03 ± 0.77#

TNF-α: tumor necrosis factor α, 8-OHdG: 8-hydroxy-2′-deoxyguanosine, iNOS: inducible nitric oxide synthase, MDA: Malondialdehyde. * Shows significant difference compared to the control group ($P<0.05$), # Shows significant difference compared to the Cd group ($P<0.05$).

Table 2. The effect of pomegranate peel extract (PPE) (500 mg/kg) and N-acetylcysteine (NAC) (100 mg/kg) alone and in combination Ornipural (1 mL/kg) treatments on some biomarkers in serum toxicity of Cd (5 mg/kg) in rats (Mean ± SEM)

Parameter	Control	Cd	Cd + PPE	Cd + PPE + Orn	Cd + NAC	Cd + NAC + Orn
TAC (U/ mL)	1.21 ± 0.16	0.24 ± 0.16*	0.39 ± 0.12	0.00 ± 0.00*	1.11 ± 0.39#	0.07 ± 0.07*
MMP2 (ng/mL)	7.06 ± 1.14	3.01 ± 1.21*	1.95 ± 0.08*	2.70 ± 0.79*	7.95 ± 0.47#	7.44 ± 0.45#
MMP9 (ng/mL)	3.66 ± 0.07	3.66 ± 0.08	3.85 ± 0.04	3.69 ± 0.10	3.71 ± 0.04	3.66 ± 0.03

TAC: total antioxidant capacity, MMP: matrix metalloprotease. * Shows significant difference compared to the control group ($P<0.05$), # Shows significant difference compared to the Cd group ($P<0.05$).

Table 3. The effect of pomegranate peel extract (PPE) (500 mg/kg) and N-acetylcysteine (NAC)(100 mg/kg) alone and in combination Ornipural (1 mL/kg) treatments on some biochemical parameters in serum toxicity of Cd (5 mg/kg) in rats (Mean ± SEM)

Parameter	Control	Cd	Cd + PPE	Cd + PPE + Orn	Cd + NAC	Cd + NAC + Orn
ALT	29.4 ± 2.22	44.6 ± 3.48*	35.25 ± 1.70	26.0 ± 2.85	31.33 ± 4.09#	32.5 ± 4.09
AST	80.0 ± 5.82	121.4 ± 10.36	88.6 ± 23.18	88.6 ± 17.07	109.0 ± 8.80	95.8 ± 1.10
ALB	34.6 ± 1.07	31.16 ± 0.83	32.0 ± 1.47	33.0 ± 1.15	33.25 ± 1.03	32.25 ± 0.25

ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALB: albumin. * Shows significant difference compared to the control group ($P<0.05$), # Shows significant difference compared to the Cd group ($P<0.05$).

Table 4. Cd levels in rat liver and kidney tissues after administration of Cd (5 mg/kg) for eight weeks (Mean ± SEM)

Gruplar	Liver Cd (µg/g)	Kidney Cd (µg/g)
Control	0.0 ± 0.0	0.0 ± 0.0
Cd	7.98 ± 1.30*	6.17 ± 3.29*
Cd + PPE	3.38 ± 1.30*, #	2.83 ± 2.31
Cd + PPE + Orn	4.78 ± 1.81*	5.01 ± 1.57*
Cd + NAC	1.89 ± 1.02#	2.26 ± 1.12#
Cd + NAC + Orn	5.64 ± 2.55*	5.46 ± 1.47*

* Shows significant difference compared to the control group (P<0.05), # Shows significant difference compared to the Cd group (P<0.05).

Table 5. The effect of pomegranate peel extract (PPE) (500 mg/kg) and N-acetylcysteine (NAC) (100 mg/kg) alone and in combination Ornipural (1 mL/kg) treatments on hematological parameters toxicity of Cd (5 mg/kg) in rats (Mean ± SEM)

Parameter	Control	Cd	Cd + PPE	Cd + PPE + Orn	Cd + NAC	Cd + NAC + Orn
WBC	7.80 ± 1.23	5.42 ± 0.79	3.52 ± 1.03	5.34 ± 1.53	4.90 ± 1.52	5.25 ± 0.25
Lymphocyte	5.04 ± 0.91	4.58 ± 0.88	2.67 ± 0.80	3.44 ± 0.86	3.18 ± 0.3	3.34 ± 0.17
Monocytes	0.28 ± 0.05	0.23 ± 0.05	0.20 ± 0.08	0.25 ± 0.07	0.15 ± 0.02	0.17 ± 0.03
RBC	8.15 ± 0.80	7.36 ± 0.60	6.87 ± 0.50	6.37 ± 1.30	7.58 ± 0.74	7.97 ± 0.15
Hb	16.02 ± 0.49	12.20 ± 1.01*	11.35 ± 0.78*	12.06 ± 2.17	10.08 ± 1.37*	12.97 ± 0.24*
HCT	45.02 ± 4.28	39.07 ± 3.27	35.47 ± 2.29	33.97 ± 6.95	39.92 ± 4.35	41.14 ± 0.80

WBC: white blood cell, RBC: red blood cell, Hb: hemoglobin, HCT: hematocrit. * Shows significant difference compared to the control group (P<0.05).

The liver MDA levels decreased in concomitant administration of NAC and NAC + Orn with Cd (P<0.05, Table 1). Ornipural has antioxidant ingredients such as L-arginine, betaine, and methionine, which positively affect antioxidant enzymes [41, 63]. N-acetylcysteine increases the level of intracellular antioxidant enzymes such as GSH and has scavenging effects on oxidative radicals [48]. The inhibition of the increase in the liver MDA level in this study may be due to the inhibition of the oxidative radicals of NAC and Orn. However, the main effect is thought to be NAC.

Lipid peroxidation is an important mechanism of cell membrane damage, and MDA is produced during the oxidative degradation of lipids [20]. It may be caused by changes in antioxidant system activities and a decrease in sulfhydryl groups (SH) and GSH level, and these formations cause Cd-induced lipid peroxidation by stimulating the prooxidative status [34]. In the current study, the serum TAC level statistically decreased in the Cd group from that of control group and its decreased level was improved by the combined use of NAC with Cd (Cd + NAC group). Total antioxidant capacity level may have decreased because Cd causes oxidative stress and its level may have improved to the normal value with the chelator and free radical scavenging effects of NAC [48, 53]. N-acetylcysteine may increase antioxidant effects by increasing the intracellular GSH level and decreasing Cd uptake into tissues [48], and inhibiting reactive oxygen species [65]. In addition to this, NAC may have caused the elimination of free oxygen radicals and decreased the liver MDA level by increasing the serum TAC level.

In this study, no statistically significant difference was observed between the groups in liver TNF-α. Cd increases the expression of inflammation-related genes by causing oxidative stress and NF-κB activation [47]. Cadmium stimulates inflammatory reactions and it has also anti-inflammatory and immunosuppressive effects [33]. The variable effects of Cd on inflammation have been reported to be dependent on dose, cell type and duration of exposure [15, 30]. Although Cd at 25, 50, and 100 mg/kg doses causes pathological damage in the intestines of mice, the TNF-α level was not altered [77]. The non-statistically significant increase in liver TNF-α levels in the Cd group can be explained by the insufficiency of the Cd dose.

8-hydroxy-2'-deoxyguanosine is used to determine DNA damage, and the presence of reactive oxygen radicals contributes to the formation of 8-OHdG [74]. In the current study, Cd may have increased liver 8-OHdG levels by causing oxidative DNA damage. N-acetylcysteine + Orn treatment may have reduced Cd-induced liver DNA damage. Antioxidant properties of Orn (especially betaine), PPE and NAC may have prevented oxidative DNA damage in the liver tissue [25, 26, 36, 41, 48, 63, 67].

The levels and activities of MMP2 and MMP9 are directly related to the remodeling of the extracellular matrix in various diseases in different tissues. Impairment of MMP activity can lead to disruption of cell homeostasis [32, 62]. Cd (at a dose of 15 ppm for 20 weeks) reduces both MMP2 and MMP9 activity [43]. In another study, prolonged Cd exposure resulted in a decrease in metallothionein and MMP2 levels and an increase in the level of MMP9 [27]. In the current study, Cd decreased the serum MMP2 level although it did not change the MMP9 level. The antioxidant and cell regeneration effects of NAC and NAC+Orn treatments may have prevented the decrease in the MMP2 level.

Cadmium causes an increase in the NO level in both hepatic and testicular tissues [6]. The NO level increases with inflammatory reactions and heavy metal toxicity [42, 46]. Nitric oxide, which is a free radical, interacts with the superoxide anion to form peroxynitrite with strong oxidant effects, and it causes further tissue damage. Cd causes an increase in the expression of iNOS and tissue damage

by the weakening of the antioxidant defense system and the production of free oxygen radicals [46]. In the current study, the liver iNOS level increased with Cd exposure, and the increase was significantly reduced by concomitant administration of NAC and PPE + Orn with Cd. The PPE and Orn combination may have reduced the level of Cd-induced liver iNOS, due to the antioxidant, and anti-inflammatory properties and synergistic effect of PPE and Orn substances [4, 37, 76]. In addition, a similar decrease in the liver iNOS level was observed by the chelator, antioxidant, and anti-inflammatory effects of NAC in the present study [13, 73]. The fact that the iNOS level did not change in the NAC+Orn group such as NAC group, because it can explain by unreduced the liver Cd level in the NAC + Orn group.

In the current study, serum ALT level significantly increased, and AST level moderately increased after Cd administration. Alanine aminotransferase is an enzyme specific to the liver parenchyma and its level is increased in cases of hepatotoxicity [61]. In hepatotoxicity induced by 5 weeks of Cd administration in rats, the levels of ALT and AST enzymes has increased. [17]. Another reason for the rise in the level of these enzymes is the stimulation of lipid peroxidation and oxidative stress [23, 40]. N-acetylcysteine treatment reduces inflammation, provides cell regeneration in the liver and decreases ALT and AST enzyme levels in a dose-dependent manner [8]. In the present study, ALT level may have improved due to decreased lipid peroxidation with NAC treatment.

In the present research, Cd levels were statistically high in liver and kidney tissues only in the Cd group, and Cd levels could not be reduced by Orn administrations. Cadmium tends to accumulate in the kidney and liver after absorption [21, 59]. It has been reported that NAC can act as a chelator on heavy metals, such as mercury, cadmium, chromium, arsenic, and gold [58]. Likewise, the flavonoids in PPE have a chelator effect on metals in general [55], and the tannins in PPE have a chelator effect on cadmium [72]. Although L-arginine in Orn is used for the treatment of kidney diseases, some researchers have stated that L-arginine and its metabolites cause kidney diseases by increasing serum potassium concentration [12, 51]. In addition, L-arginine is converted to citrulline, which accumulates in the renal tubules and then causes tubular disorders [51]. In the current study, NAC alone and PPE treatments may have reduced Cd levels in kidney and liver tissues with their chelator effects. The lack of the same effect in combination treatments with Orn may be due to the L-arginine in its structure; it may have disrupted tubular structure with citrulline accumulation and decreased Cd excretion.

The Hb level may decrease with the decrease in the amount of iron in the blood [2] and the increase in erythrocyte destruction [22]. In the current study, Cd administration caused a decrease in the level of hematological parameters, especially in Hb concentration. This can be explained by the passage of immature erythrocytes into the blood as a result of Cd suppressing the bone marrow [10]. On the other hand, it suggests that the current treatments protocols against Cd do not cause a significant change in blood parameters and they can be used safely cadmium toxication.

In conclusion, Cd is a major important pollutant especially for the human and animal health and its toxic effects should minimized. It was determined that the best treatment option was the use of NAC in the current research. In addition, it has been observed that PPE and NAC+Orn treatments prevent the osteoporotic changes caused by according to the results of the histopathological and morphological research (unpublished data). Therefore, NAC can be effective in reducing the level of Cd in human/animal and eliminating the toxic effects of Cd, whereas further research need on the effects of PPE and Orn combinations for the treatment of cadmium toxicity. In future studies, these treatment options should investigated with different doses and chelators for cadmium toxicity.

CONFLICT OF INTERESTS. The authors declared that there is no conflict of interest.

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