Oleuropein Ameliorates Cisplatin-induced Hematological Damages Via Restraining Oxidative Stress and DNA Injury

Fatime Geyikoğlu¹ · Suat Çolak² · Hasan Türkez^{3,4} · Murat Bakır¹ ·
Kübra Koç¹ · Mir Khalil Hosseinigouzdagani¹ · Salim Çerig¹ · Merve Sönmez⁵

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Abstract The prevalence of cancer, in the world is increasing steadily. Despite intense research efforts, no approved therapy is yet available. Cisplatin is a chemotherapeutic drug but induces acute tissue injury. Oleuropein (OLE) is a major phenolic compound and used as a possible natural antioxidant, antimicrobial, and anti-cancer agent. We hypothesized that antioxidant activity of OLE may decrease cisplatin-induced oxidative stress and prevent to the development of chemotherapeutic complications including abnormality in hematological condition. Male Sprague Dawley rats were used in the experiments. Rats were randomly assigned to one of eight groups: control group; group treated with i.p. injection in a single dose of 7 mg/kg/day cisplatin; groups treated with 50, 100 and 200 mg/kg/day OLE (i.p.); and groups treated with OLE for 3 days starting at 24 h following cisplatin injection. First, hematological assessment was appreciated between control and experimental groups. Second, total oxidative stress (TOS) and total antioxidant capacity (TAC) levels of blood were measured by biochemical studies. In addition to this, oxidative DNA damage was

determined by measuring as increases in 8-hydroxy-deoxyguanosine (8-OH-dG) adducts. The treatment with cisplatin elevated the TOS and 8-OH-dG levels that were then reversed by OLE. Reductions in antioxidant capacity with respect to corresponding controls were also restored by OLE treatment. These findings suggest that the OLE treatment against cisplatin-induced toxicity improves the function of blood cells and helps them to survive in the belligerent environment created by free radicals.

Keywords DNA damage · Oxidative stress · Total antioxidant capacity · Hematological parameters · Oleuropein · Cisplatin

Introduction

Conventional chemotherapy agents that aim to kill tumor cells may also damage normal cells and thus result in severe side-effects [1]. Cisplatin is one of the most effective anticancer drugs for treatment of testicular, ovarian, and cervical cancers as well as many types of other solid tumors such as head and neck cancers [2, 3]. But its clinical application has been limited because of the onset of severe side effects such as renal insufficiency, peripheral and central neuropathies, and also vascular complications [4, 5]. Tubular apoptosis and the peripheral blood mononuclear cell apoptosis is recognized as a major pathogenic factor in cisplatin-induced renal and hematological toxicity [6, 7].

Cisplatin-induced cell death may involve increased production of reactive oxygen species (ROS) and oxidative stress [8]. Peroxyl radical and lipid peroxides generated during lipid peroxidation mutilate cellular components and affect erythrocyte function in blood [9]. Hydroxyl radicals

✉ Fatime Geyikoğlu
geyikogluff@yahoo.com

¹ Department of Biology, Faculty of Science, Atatürk University, Erzurum, Turkey

² Üzümlü Vocational School, Erzincan University, Erzincan, Turkey

³ Department of Molecular Biology and Genetics, Faculty of Science, Erzurum Technical University, Erzurum, Turkey

⁴ Department of Pharmacy, University “G. d’Annunzio” Chieti-Pescara, Chieti, Italy

⁵ Department of Biology, Faculty of Art and Science, Erzincan University, Erzincan, Turkey

induce oxidative DNA damage, which alter purine and pyrimidine bases generating 8-OH-dG that induce G to T transversions in DNA [10, 11]. Many strategies have been applied for reduction of cisplatin-induced tissue toxicity in animal models and humans including administration of L-arginine [12] and antioxidant agents such as vitamin C and E [13], melatonin [14] N-acetyl cysteine [15], and herbal extracts [16, 17].

Numerous studies have now documented the beneficial effects of various antioxidants in cisplatin-induced toxicity. Olive polyphenols are recognized as potential antioxidant additives for food, dietary supplements, functional foods and natural cosmetics as well as for pharmaceutical industries [18, 19]. OLE, one of the most bioactive polyphenols present in the extra virgin olive oil could provide a protective and therapeutic effect against a number of pathologies, including Alzheimer's disease as well as obesity, type 2 diabetes, non-alcoholic hepatitis, and other natural or experimentally induced pathological conditions [20–22]. In particular, the recent data suggest that the OLE presents are able to scavenge free radicals and afford an adequate protection against peroxidation [23].

Over the past decade, intense focus has been dedicated on investigating processes involved in the toxicity of anticancer drugs. It is lack of effective drugs to remedy cisplatin-induced hematotoxicity at present [24]. As oxidative stress plays the major role in the pathogenesis of cisplatin-induced toxicity and the antioxidant effect of OLE is well known, we hypothesized that OLE may be beneficial in preventing the cisplatin-induced hematotoxicity. Therefore, we aimed to evaluate the therapeutic potential of OLE in cisplatin-mediated hematological damages by measuring TOS and TAC levels, which are important markers for lipid peroxidation and antioxidative capacity. Furthermore, the present study was carried out to reveal 8-OH-dG, the hallmark of oxidative DNA damage in order to better understand the protection mechanism of OLE on blood lymphocytes.

Materials and Methods

Animals

Fifty six adult male Sprague-Dawley rats (70 days old, weighing 190–220 g) obtained from Medical Experimental Application and Research Center, Atatürk University were used. Animals were housed in a large metal cages in an air-conditioned room (22 ± 2 °C) with 12 h light–dark cycle. Standard rat feed and water were provided ad libitum. The rats were allowed to acclimatize to the laboratory environment for 7 days before the start of the experiment. All procedures were performed

in conformity with the Institutional Ethical Committee for Animal Care and Use at Atatürk University (protocol number: 2014/8-144) and the Guide for the Care and Use of Laboratory Animals [25].

Experimental Protocol

The rats were weighed and randomly allocated into eight experimental groups: (1) control: the animals received 1 mL of distilled water as vehicle; (2) Cisplatin (Sigma Chemical Co., St. Louis, MO, USA): the animals received 7 mg Cisplatin/kg b.w., diluted in distilled water (1 mL); (3) OLE (HPLC grade ≥ 98 %; Sigma): the animals received 1 mL of OLE solution; and (4) Cisplatin/OLE the animals received 1 mL of preparations of OLE following cisplatin administration. The injections of cisplatin were given using a single dose, via intraperitoneal route for 24 h. The OLE groups received intraperitoneal injections with a daily single dose of OLE (50, 100 and 200 mg/kg/day) for a total period of 3 days. On day 4 after injections, the rats were fasted overnight and then anesthetized with ether. Blood samples were intracardially collected in BD Vacutainer® Blood Collection Tubes with EDTA (BD: Becton, Dickinson and Company) for hematological parameters. At the end of this application, rats were immediately sacrificed by cervical decapitation.

Hematology

Blood was collected in EDTA vials. Routine hematological parameters such as RBC (Hb, Ht, MCV, MCH, MCHC), WBC (basophil, monocyte, eosinophil, lymphocyte and neutrophil) and platelet count were analyzed using automated Vet ABC Animal blood counter (ABX Diagnostics, France).

Biochemical Assays

The plasma was separated by centrifugation and used for TAC and TOS measurements.

Measurement of TAC

TAC was measured using a novel automated colorimetric measurement method developed by Erel [26]. In this method the hydroxyl radical, the most potent biological radical, is produced by the Fenton reaction and reacts with the colorless substrate *O*-dianisidine to produce the dianisyl radical, which is bright yellowish-brown in color. Upon the addition of a plasma sample, the oxidative reactions initiated by the hydroxyl radicals present in the reaction mix are suppressed by the antioxidant components of the plasma, preventing the color change and thereby providing

an effective measure of the TAC of the plasma. The assay results are expressed as mmol Trolox equiv/L.

Measurement of TOS

TOS was measured using a novel automated colorimetric measurement method developed by Erel [27]. In this method, oxidants present in the sample oxidize the ferrous ion-odanisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules (lipids, proteins) present in the sample. The assay is calibrated with hydrogen peroxide, and the results are expressed in terms of micromolar hydrogen peroxide equivalent per liter ($\mu\text{mol H}_2\text{O}_2$ equiv/L).

Measurement of 8-OH-dG

The lymphocytes were separated from peripheral blood by using Ficoll reagent. 8-OHdG content was detected with the 8-OHdG enzyme-linked immunosorbent assay kit (ELISA, USCN Life Sciences, Wuhan, China). DNA was extracted from 10^6 lymphocytes. Approximately 500 μg extracted DNA was digested with nuclease P1 (1 U) and acid phosphatase (1 U) in a 10 mM sodium acetate solution. After incubation at 37 °C for 90 min, the mixture was centrifuged twice at $10,000\times g$ for 15 min each, and the supernatant was used to measure 8-OH-dG levels. Each DNA sample was measured in duplicate, and level of 8-OH-dG expressed as pg/mg DNA [28].

Statistical Analysis

For statistical analysis, we used SPSS for Windows 18.0 (SPSS Inc., Chicago, USA). The experimental data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey post hoc test for multiple comparisons. Results are presented as mean $\pm$ standard deviation (SD) and p values <0.05 were regarded as statistically significant.

Results

According to our results, treatment of rats with cisplatin caused a significant decrease in hematological values. The 50 mg/kg dose of OLE on RBCs, Hb, Ht, MCV, MCH and MCHC values has not any positive effect. Besides, the low dose of OLE insufficiently enhanced the number of WBCs, basophil, monocyte, eosinophil, lymphocyte, neutrophil

and platelet. Whereas, the treatment with 100 mg/kg OLE significantly reduced the adverse effects of cisplatin ($p < 0.05$) and the hematological parameters showed an increase to near normal level after administration of OLE. Our results also indicated that the efficacy of supplementation 200 mg/kg OLE on these parameters was similar to the effects of 100 mg/kg OLE dose (Tables 1, 2).

Table 3 shows the effects of cisplatin and OLE on biochemical parameters in all experimental groups. As compared with the controls, the TAC levels were markedly decreased in cisplatin-treated rats while TOS increased ($p < 0.05$). In alone groups, the OLE slightly increased the level of TAC at 50 mg/kg dosage but the best results were observed at the doses of 100 and 200 mg/kg OLE. In CIS plus 100 mg/kg OLE groups, TAC increases were significant statistically as compared with cisplatin groups and oxidative stress returned to the control levels. However, the effects of OLE on cell defense system did not show increasing dose–response relationship.

The levels of 8-OH-dG were measured using an 8-OH-dG detection kit. There were no significant differences between the levels of 8-OH-dG in the control and all OLE treated groups (Table 4). On the contrary, the level of 8-OH-dG was significantly higher in cisplatin groups as compared to control group. However, increasing doses of OLE decreased cisplatin-induced 8-OH-dG amounts and oxidative DNA damages significantly were prevented (100 and 200 mg/kg OLE) ($p < 0.05$) (Table 4).

Discussion

The functional relationship between chemotherapy drugs and oxidative stress has been reported for several decades and remains an important focus of study. Chemotherapy with cisplatin includes anemia, thrombocytopenia, and leukopenia [29]. Following treatment with cisplatin in present study, hematological changes comprise decreased erythrocyte counts, Hb and Ht values, as well as decreased MCV, MCH and reduced MCHC, suggestive of a microcytic hypochromic anemia. In this type of cisplatin-induced anemia above hematological parameters decreases as a result of suppression of erythropoiesis [30]. Our study shows that increased oxidative stress might promote this pathological status progression in cisplatin-based chemotherapy.

Cisplatin causes the generation of reactive oxygen species (ROS) and to inhibit the activity of antioxidant enzymes in the blood tissue. It is reported that ROS increase hemoglobin glycation and erythrocyte fragility [31, 32]. More specifically hemoglobin-derived iron might contribute to the pathogenesis of cisplatin by inducing oxidative stress [33, 34]. Thus, cisplatin-induced Hb

Table 1 The effects of CIS and OLE on red blood cells and values in rats

Groups	RBC ($10^6/\mu\text{L}$)	Hb (g/dL)	Ht (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)
Control	11.7 ± 0.71^b	13.52 ± 0.97^b	51.27 ± 2.77^b	56.43 ± 3.17^b	20.14 ± 1.55^b	33.05 ± 3.63^b
CIS	4.86 ± 0.13^a	5.98 ± 0.51^a	41.35 ± 1.93^a	38.74 ± 2.75^a	9.11 ± 1.67^a	22.15 ± 2.01^a
OLE 50	10.9 ± 0.70^b	13.02 ± 0.82^b	51.46 ± 3.12^b	55.61 ± 2.68^b	20.52 ± 2.06^b	33.22 ± 3.27^b
OLE 100	11.2 ± 0.61^b	12.92 ± 1.02^b	50.09 ± 2.07^b	55.20 ± 1.73^b	19.84 ± 1.77^b	32.66 ± 4.03^b
OLE 200	11.8 ± 0.88^b	13.33 ± 1.08^b	50.55 ± 1.99^b	56.05 ± 3.01^b	20.33 ± 1.70^b	33.91 ± 2.88^b
CIS + OLE 50	5.12 ± 0.66^a	6.43 ± 0.92^a	43.02 ± 2.08^a	43.51 ± 3.22^a	10.63 ± 1.05^a	23.11 ± 1.77^a
CIS + OLE 100	10.58 ± 0.86^b	13.05 ± 0.80^b	50.71 ± 2.92^b	54.95 ± 2.45^b	19.68 ± 1.83^b	31.45 ± 3.07^b
CIS + OLE 200	9.21 ± 1.17^b	12.47 ± 1.32^b	49.65 ± 2.27^b	54.01 ± 3.61^b	18.08 ± 2.55^b	30.81 ± 2.49^b

Values are mean $\pm$ SD of 7 rats in each group. Column means labeled with different letters (a, b) were significantly different ($p < 0.05$)

CIS, Cisplatin; OLE, oleuropein

Table 2 The effects of CIS and OLE on white blood cell types

Groups	WBC ($10^3/\mu\text{L}$)	Basophil (%)	Monocyte (%)	Eosinophil (%)	Lymphocyte (%)	Neutrophil (%)	Platelet ($10^3/\mu\text{L}$)
Control	8.27 ± 0.41^b	0.92 ± 0.03^b	17.12 ± 1.47^b	7.86 ± 1.02^b	67.93 ± 4.26^b	33.31 ± 2.67^b	896.65 ± 29.12^b
CIS	2.21 ± 0.53^a	0.17 ± 0.02^a	5.28 ± 1.11^a	1.82 ± 0.28^a	26.51 ± 2.04^a	$12.75 \pm 1.91^{*a}$	319.08 ± 6.82^a
OLE 50	8.11 ± 1.17^b	0.97 ± 0.07^b	17.17 ± 1.51^b	7.60 ± 0.81^b	66.11 ± 3.71^b	33.64 ± 1.90^b	863.54 ± 36.20^b
OLE100	8.39 ± 0.98^b	0.89 ± 0.06^b	17.01 ± 1.37^b	7.96 ± 1.01^b	66.87 ± 4.16^b	32.69 ± 2.82^b	877.65 ± 27.33^b
OLE200	8.19 ± 0.83^b	0.91 ± 0.03^b	16.89 ± 2.03^b	7.74 ± 1.06^b	67.44 ± 3.40^b	32.75 ± 3.12^b	907.23 ± 21.79^b
CIS + OLE 50	3.78 ± 0.79^a	0.23 ± 0.08^a	7.20 ± 1.08^a	2.09 ± 0.37^a	31.24 ± 2.17^a	$13.63 \pm 1.26^{*a}$	385.62 ± 9.03^a
CIS + OLE100	7.64 ± 0.65^b	0.90 ± 0.02^b	16.01 ± 0.98^b	7.18 ± 0.70^b	63.87 ± 2.19^b	31.54 ± 1.71^b	818.06 ± 5.73^b
CIS + OLE200	8.14 ± 0.96^b	0.87 ± 0.02^b	16.34 ± 0.82^b	6.79 ± 0.49^b	66.04 ± 4.13^b	32.58 ± 2.03^b	852.01 ± 14.44^b

Values are mean $\pm$ SD of 7 rats in each group. Column means labeled with different letters (a, b) were significantly different ($p < 0.05$)

CIS, Cisplatin; OLE, oleuropein

Table 3 The effects of CIS and OLE on TAC and TOS levels in rats

Treatments (mg/kg bw)	TAC (Trolox equiv/mmol/L)	TOS (H_2O_2 equiv/ $\mu\text{mol/L}$)
C	14.22 ± 0.64^b	1.91 ± 0.17^b
CIS	3.85 ± 1.72^a	8.74 ± 0.92^a
OLE 50	15.83 ± 1.03^b	2.15 ± 0.13^b
OLE 100	16.20 ± 1.44^b	2.01 ± 0.29^b
OLE 200	15.96 ± 1.93^b	2.11 ± 0.19^b
CIS + OLE 50	4.14 ± 0.95^a	7.63 ± 0.88^a
CIS + OLE 100	12.17 ± 1.03^b	2.92 ± 0.34^b
CIS + OLE 200	13.32 ± 1.66^b	3.14 ± 0.27^b

Values are mean $\pm$ SD of 7 rats in each group. Column means labeled with different letters (a, b) were significantly different ($p < 0.05$)

CIS, Cisplatin; OLE, oleuropein

reduction is related to suppression of erythropoiesis and iron supply to erythroblasts [33, 35]. We also establish that one of the effects of cisplatin is the production of erythrocytes with lower MCV, MCH and MCHC and these parameters are closely related to Ht levels and Hb. Hb and Ht data could be strongly influenced by MCV, MCH and MCHC values [36]. The fall of Ht is a reason for the

decreased erythrocyte number [37]. The erythrocytes are a convenient model to understand the subsequent oxidative deterioration of biological macromolecules. Like other biological membranes, the RBC membrane is susceptible to lipid peroxidation under oxidative stress. RBCs are oxygen carriers with a high poly unsaturated fatty acid (PUFA) content on their membranes and a high

Table 4 The effects of CIS and OLE on 8-OHd-G levels in lymphocytes of rats

Groups	8-OH-dG level (as pg/mL)
Control	0.95 ± 0.02 ^b
CIS	5.38 ± 0.31 ^a
OLE 50 mg/kg	0.90 ± 0.02 ^b
OLE 100 mg/kg	1.11 ± 0.09 ^b
OLE 200 mg/kg	1.03 ± 0.02 ^b
CIS + OLE 50 mg/kg	4.94 ± 0.21 ^a
CIS + OLE 100 mg/kg	1.55 ± 0.03 ^b
CIS + OLE 200 mg/kg	1.42 ± 0.06 ^b

Values are mean ± SD of 7 rats in each group. Column means labeled with different letters (a, b) were significantly different ($p < 0.05$)

CIS, Cisplatin; OLE, oleuropein

concentration of cellular hemoglobin, and are therefore particularly exposed to oxidative damage [38]. High oxygen tension, PUFA and iron are potent catalysts for the radical reactions, rendering erythrocytes readily susceptible to both extracellular and intracellular sources of ROS, which induce lipid peroxidation and thereby cause membrane malfunction by altering their fluidity and membrane-bound enzyme and receptor functions [39]. However, supplementation of antioxidants may significantly modulate the induced changes in LPO level, total lipids, total ATPases, glutathione, and antioxidant enzymes in erythrocytes [40].

Various free radical scavengers have been shown to be effective in protection from cisplatin-induced blood toxicity and treatment with such agents provides significant protection against cisplatin-induced acute hematologic disorders [41]. In our study, OLE administration may prevent cisplatin-induced anemia in rat by its free radical-trapping activity (decreased TOS). A significant effect is detected on Hb, MCV, MCH, MCHC, and Ht values of cisplatin-received group after administration of 100 mg/kg OLE. Furthermore, it is shown that OLE at doses of 200 mg/kg provides similar effects on these parameters but 50 mg/kg OLE does not show any positive effect. OLE is an antioxidant and strong free radical scavenger [24]. Its antioxidant property is useful for the protection of cellular macromolecules from oxidative damage induced by different agents. In vitro studies have shown that the antioxidant activity of OLE protects different cell types such as CaCo-2 [42], erythrocytes [43], and PC12 [44] from hydrogen peroxide (H₂O₂)-induced cytotoxicity as evidenced by several methods like the leakage of lactate dehydrogenase and the 3-[4,5-dimethyl(thiazol-2-yl)]-3,5-diphenyltetrazolium bromide assay. Besides, OLE in vivo

systems has been shown to significantly attenuate the increase of lipid peroxidation against ethanol-induced gastric ulcers via elevation of antioxidant enzyme activities in rats [45].

The cisplatin disturbs redox status intensively and leads to oxidative stress in human leukemic cell line. Thus, it decreases leukocytes and platelets in patients received chemotherapy [46]. Our observations indicate that the antioxidant property of 100 mg/kg OLE (increased TAC) provides a significant protection against this frequent complication of cisplatin-based chemotherapy. Our results agree with those published by Fabiani et al. [47], who found a significant protective effect on human blood leukocytes by OLE. OLE can be regarded as typical phenolic antioxidant. It is suggested that this antioxidant may be able to reduce leukocyte lipoxygenase enzymes and the damaging consequences of their ability to release ROS whilst leaving unimpaired the generation of prostaglandins, which promote microvascular blood flow and act as immune-modulators. It is also natural oxygen radical scavenger that reduce thrombin-induced protein tyrosine phosphorylation and Ca²⁺ signaling. Thus, OLE may prevent thrombotic complications associated to platelet hyperaggregability and be the base for the development of anti-aggregate therapeutic strategies. Nevertheless, these comments are not based on full dose–response studies, and a more accurate investigation of the relative properties of these olive oil phenolic as biological antioxidants will be worthwhile [48, 49]. We report that OLE administration dose dependently increases leucocyte and thrombocyte count (50 and 100 mg/kg OLE). However, increasing dose response pattern is not demonstrated on studied parameters. As a matter of fact, the 200 mg/kg dose of OLE does not allow for a better evaluation of therapeutic effect because of its unchanged antioxidant property.

In the present study, the antioxidant capacity in cisplatin treated group shows a marked decline while TOS and 8-OH-dG levels are significantly higher when compared with the control groups. According to these results, it is clear that oxidative stress induced by cisplatin reduces the 8-OH-dG-mediated DNA methylation and activity of antioxidant enzymes. Our findings are consistent with the reports of investigators [50–52]. On the other hand, OLE can attenuate oxidative stress mediated damage of DNA in human blood mononuclear cells and HL60 cells [46]. Our data supplement this information since enhanced antioxidant activity prevents oxidative stress, which in turn reduces 8-OH-dG levels in lymphocytes of cisplatin-treated animals.

Based on our results, it can be concluded that OLE has geno-protective feature in the cisplatin-induced genotoxicity and can be considered a potential candidate to protect blood cells against the deleterious effect of oxidative DNA

damages in chemotherapy. Furthermore, this *in vivo* evaluation has provided beneficial results for the use of OLE in future clinical trials.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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