Clinical and Experimental Health Sciences

Pomegranate Peel Extract Reduces Cisplatin-Induced Toxicity and Oxidative Stress in Primary Neuron Culture

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ABSTRACT

Objective: Cancer is the most common cause of death after cardiovascular diseases. Cisplatin used in most types of cancer produces neurotoxicity. In this study, we aimed to investigate the effects of pomegranate peel extract (1) in different doses, as potent antioxidants, on the prevention of neurotoxicity due to cisplatin, which is frequently used in cancer treatment.

Methods: In our study, newborn rat cortex was used. 2 hours following the application of PPE at 200, 300 and 400 mg/mL, neurotoxicity was established by applying cisplatin in 50 and 100 µM concentrations.

Results: In our study, cisplatin decreased cell viability in increasing doses, while PPE showed the best neuroprotective effect in high doses. Increased total oxidant capacity due to toxicity was significantly improved by PPE4. The antioxidant capacity decreased in the toxicity group showed improvement with the administration of PPE4. At the same time, increased TNF-α mRNA expression after cisplatin administration was significantly reduced with the administration of PPE4. The increased caspase 3 (CAS 3) and caspase 9 (CAS 9) mRNA expression due to cisplatin showed improvement with the administration of PPE4.

Conclusion: These results indicated that PPE could inhibit cisplatin-induced neurotoxicity, and these effects may be related to anti-apoptotic and antioxidants activities.

Keywords: Pomegranate, antioxidant, cisplatin, neurotoxicity, primary neuron

1. INTRODUCTION

Cancer is the second most common cause of death after cardiovascular diseases. The morbidity and mortality rates are increasing day by day. Antineoplastic agents are frequently used as the most effective treatment protocol (2).

Cisplatin is a chemotherapeutic agent used commonly in the treatment of solid tumors in various organs. The clinical use of cisplatin is limited due to its serious side effects on the nervous system (3). In addition, dose-limiting factors such as epilepsy, stroke and neuropathy, changes in consciousness, cerebral violations and ototoxicity are observed. All these side effects are often dose-limiting factors (4). Additionally, oxidative damage, inflammation, mitochondrial dysfunction, DNA damage, and apoptosis are involved in the mechanism of cisplatin neurotoxicity (4). Anticancer drugs, mainly ATPase dependent Na_+/K_+ pumps and Ca^{2+} homeostasis, break down mitochondria. Accumulation of dysfunctional mitochondria causes oxidative stress and peripheral nerve damage (5). Based on this information, studies have shown that cisplatin has a toxic effect by a mechanism caused by oxidative damage.

In shortly, oxidative stress has been recognized as an imbalance between the production of free radicals and antioxidant defense mechanisms that potentially lead to tissue damage. Oxidative stress plays a key role in the development of cerebrovascular and/or neurodegenerative diseases through different molecular pathways (6). Neurons are more sensitive to oxidative damage than other cells. The reasons for this are known to be their high oxygen consumption and low antioxidant enzyme activities etc. (6). From this point of view, it has been of interest to the researchers to search for neuroprotective drugs of natural origin against the neuronal death caused by oxidative stress. The prevention of chemotherapy-induced neurotoxicity associated with the clinical use of cisplatin is thus far an unsolved issue. Neurotoxicity is known to occur as a result of antineoplastic agents managing neuron cells to apoptosis. However, the effects of antineoplastic agents can be permanent in patients after chemotherapy and adversely affect the life of the patient after treatment. Therefore, today both clinical and preclinical studies are being conducted to prevent this neurotoxicity.

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Punica granatum, an important member of the *Punicaceae* family is a fruit with many features known since ancient times. Pomegranateisaperennialherbofthegenus*Punicagranatum* which is included in the genealogy family (7). Pomegranate peel, seed and fruit have been used for therapeutic purposes for years. Pomegranate peel is rich in 5 phenolic compounds, mainly flavanoids (anthocyanin, catechin and other complex flavanoids) and tannins (punicalin, punicalagin, gallic acid and ellagic acid) (8). Phenolic compounds are natural compounds with antioxidant activity. Proven in-vitro testing is available using four separate testing methods, showing that pomegranate juice and seed extracts have 2-3 times the antioxidant capacity of red wine or green tea (9). In additional in-vivo studies, pomegranate extracts have been shown to clear free radicals, reduce macrophage oxidative stress and lipid peroxidation (10). It has also been clinically indicated to increase plasma antioxidant capacity in older people (11).

Therefore, it was aimed to determine the effects of pomegranate peel extract, which is a natural antioxidant, and an easy to reach product with strong antioxidant activity, on neurotoxicity due to cisplatin.

2. METHODS

2.1. Preparation of Standard and Sample Solutions

Gallic acid, punicalagin A&B, and ellagic acid stock solutions were prepared with methanol at 1.00 mg/mL concentrations and working solutions were obtained by diluting stock solutions with phosphate buffer solution (pH=2.5, 0.025 M) to relevant concentration levels.

Pomegranate bark extract was solubilized with methanol and centrifuged at 10 000 rpm for 5 minutes. The supernatant was diluted with phosphate buffer solution and filtered through HDPE syringe filter with 0.45 µm pore size before injection.

2.2. Chromatographic Conditions

Method development and sample analysis were achieved with 1260 series Agilent HPLC equipped with auto sampler injector, diod array detector (DAD), column thermostat, quaternary pump compartments and Chemstation data acquisition program (Agilent Technologies, Santa Clara, CA, USA). Elution of the compounds were obtained by Kinetex RP_{C18} column (4 µm particle size, 3.9x250 mm I.D., store up at 25 °C), 5.00 µL injection volume. The mobile phase in channel (A) 0.025 M KH_2PO_4 buffer adjusted at pH=2.5 with phosphoric acid and in channel (B) acetonitrile and the gradient elution profile was: 0-10 minutes from 15% to 25% of B, 10-15 mintes from 25% to 60% of B with 0.5 mL/min flowrate. Detection performed at 254 nm for ellagic acid, 262 nm for punicalagin A&B and 272 nm for gallic acid DAD wavelengths.

The method was validated according to the International Council for Harmonization (ICH) guideline. Identification of the peaks were provided by comparison of retention times and DAD spectra of standard compounds and extract samples (Figure 1). Validation parameters and results are summarized in Table 1. Quantification of the pomegranate bark extracts was obtained by injection of three aliquots of extract solution to the system.

Table 1. *Chromatographic Parameters*

Compound	Gallic acid	Punicalagin A	Punicalagin B	Ellagic acid
Wavelength (nm)	272	262	262	254
Retention time (minute)	7.77 ± 0.08	9.56 ± 0.04	10.65 ± 0.05	16.54 ± 0.08
LOD (mg/L)	2.12	1.53	1.45	0.94
LOQ (mg/L)	2.56	1.95	1.85	1.29
Linear Concentration Range (mg/L)	5.00–50.00	40.00–400.00	50.00–500.00	10.00–150.00
Regression Equation (y= mx + b)	0.14x+8.87	0.01x-4.33	0.02x+11.55	0.06-3.97
Regression Coefficient (R^2)	0.9984	0.9996	0.9996	0.9999
Average Recovery	100.54 %	101.47 %	102.18 %	98.92 %
RSD% Peak Areas of Inter-Day Precision (n=3)	2.94	3.12	4.19	0.49
RSD% Peak Areas of Intra-Day Precision (n=9)	3.29	3.85	5.18	1.13

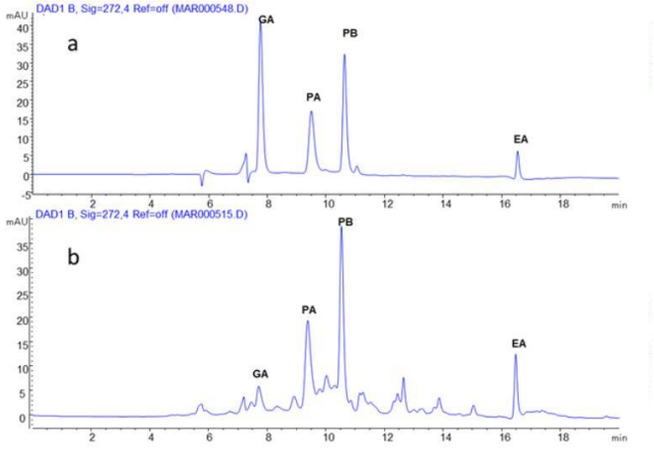

Figure 1. *Chromatograms of (a) standard compounds; (b) pomegranate bark extracts at 272 nm wavelength (GA: Gallic acid, PA: Punicalagin A, PB: Punicalagin B, EA: Ellagic acid)*

2.3. Animal Supply and Ethics Committee

This study was conducted with permission from the Local Ethics Committee of XX University Animal Experiments dated 25.01.2016 numbered 2016–016. A total of 10 newborn

Spraque Dawley rats obtained from the experimental animal laboratory were used.

2.4. Primary Neuron Culture

Primary neuron culture was conducted in the same way as described at our previous study (12). Pomegranate peel extract (1) at 200, 300 and 400 mg/mL concentrations was administered 2 hours before the onset of toxicity. The working order is summarized in Figure 2.

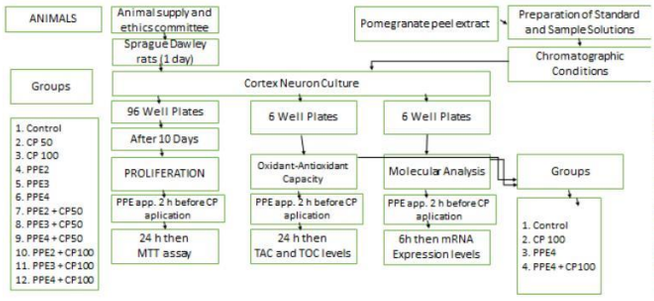

Figure 2. *Description chart of the primary neuron culture experiment*

Experimental groups; 1. Control, 2. 50 µM (C50), 3. 100 µM (C100), 4. PPE2 (200 mg/mL), 5. PPE3 (300 mg/mL), 6. PPE4 (400 mg/mL), 7. PPE2 + C50, 8. PPE3 + C50, 9. PPE4 + C50, 10. PPE2 + C100, 11. PPE3 + C100, 12. PPE4 + C100

2.5. Proliferation Assay

Cell viability was determined by MTT (3-(4,5-dimethylthiazol – 2-yl)-2,5-diphenyltetrazolium bromide) proliferation kit (Cayman Chemical, Ann Arbor, Mi, USA) described as previously (12, 13). 5 wells were used for each group.

2.6. Total Antioxidant Capacity (TAC) and Total Oxidant Capacity (TOC) Analysis

In cisplatin toxicity, commercial kit was used to determine the TAC and TOC levels of neuroprotective endogenous molecule PPE on primary neuron culture cells (Rel Assay Diagnostics, Gaziantep, Turkey). 5 wells were used for each group. The kit application was calibrated with a stable antioxidant, vitamin E analog and called Trolox equivalent (14). A commercial TOC kit was used to determine the TOC levels of neuroprotective PPE on primary neuronal culture cells in cisplatin toxicity (Rel Assay Diagnostics, Gaziantep, Turkey).

2.7. Molecular analysis

For RT PCR analysis, 4 groups (Control, Cisplatin 100 µM, PPE 400 mg/mL, PPE 400 mg/mL Cisplatin 100 µM) were created to work on the most effective doses according to MTT and TAC/TOC levels. 5 wells were used for each group. In our study, the expression levels of mRNA, CAS 3, CAS 9 and TNF-α, were compared between groups. The most toxic dose for cisplatin (C100) and the most effective dose for pomegranate (PPE 400) were preferred when determining real time PCR groups. mRNA extraction and complementary DNA (cDNA) synthesis were performed according to the methods described in our previous studies (15). mRNA extraction was performed from previously homogenized cells (pooled, 20 mg). Total mRNA was purified on the QIACUBE (Qiagen, Hilden, Germany) device according to the manufacturer's instructions using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA samples were reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA).

Relative mRNA expression analyzes of caspase-3 (CAS 3), caspase-9 (CAS 9) and tumor necrosis factor-alpha (TNF-α) from cDNAs obtained from RNAs of cell was performed on StepOne Plus Real Time PCR instrument (Applied Biosystems) using the Taqman Gene Expression kit (Applied Biosystems). β-actin was used as housekeeping gene. All data were expressed as fold change in expressions compared to the control group using the $2^{-\Delta\Delta Ct}$ method.

2.8. Statistical Analysis

The results of our studies were statistically evaluated by IBM SPSS 20 package program. The groups were checked primarily by Skewness and Kurtosis tests to see if they fit the normal distribution. Kruskal-Wallis test was used for analyses of the non-parametric data as they do not conform to normal distribution. $P<0.05$ was considered significant. Median and range values were used for graphics.

3. RESULTS

3.1. HPLC-DAD Analysis of Contents in PPE

HPLC-DAD system was used for identification and quantification of individual phenolic compounds of PPE. Figure 1 shows standard compounds and extract samples containing sharp, symmetrical and well-resolved peaks that were observed in four compounds. The corresponding result is given in Table 1, which indicates that PPE amounts were calculated as 14.45±0.53 mg/g, 191.56± 0.36 mg/g, 189.48± 0.62 mg/g, 68.02± 0.42 mg/g for gallic acid, punicalagin A, punicalagin B and ellagic acid respectively.

3.2. Proliferation Assay Results

Primary neuron cells were treated with PPE 200, PPE 300, PPE 400 mg/mL, and cisplatin 50 and 100 µM dosing together and/or alone. After application of PPE for 2 h, cisplatin toxicity was determined and MTT test was applied. According to the results of our study, the dose of PPE 300 and PPE 400 mg/mL increased cell viability significantly when compared to the control group ($p<0.01$). It was determined that cell viability decreased with cisplatin application only. PPE3 and PPE4 were able to prevent toxicity against low-dose and high-dose cisplatin-induced toxicity ($p<0.001$) (Figure 2).

3.3. Antioxidant and Oxidant Results

Neurotoxicity with cisplatin: According to the total antioxidant capacity (TAC) and total oxidant capacity (TOC) measurements, in neuron culture medium exposed to PPE 400 mg/mL for 2 h and then cisplatin for 24 h, and there was statistically significant decrease in TAC and statistically significant increase in TOC measurements when compared with the control groups. As shown in Figure 3, cisplatin administration significantly decreased TAC level compared to control. With the application of PPE4, the decreased TAC level increased significantly compared to the control ($p<0.05$). On the contrary, increased TOC level by cisplatin application decreased significantly compared to control with PPE4 administration ($p<0.01$) (Figure 4).

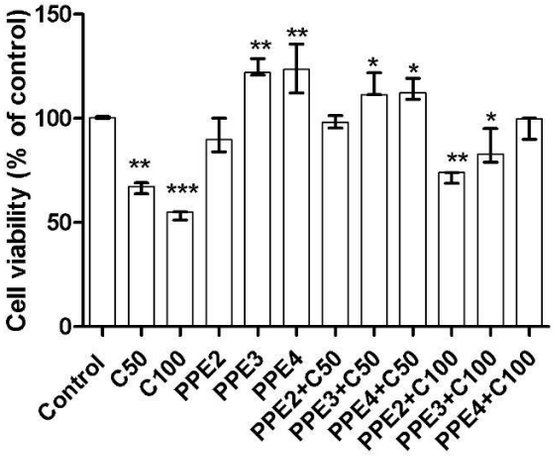

Figure 3. *The effects of PPE against cisplatin toxicity on cell proliferation in neuron cell line in 24 hours. C50: 50 µM Cisplatin; C100: 100 µM Cisplatin; PPE2: Punica granatum peel extract 200 mg/mL; PPE3: Punica granatum peel extract 300 mg/mL; PPE4: Punica granatum peel extract 400 mg/mL; error bars represent standard deviation; *p<0.05; **p<0.01; ***p<0.001 versus control groups.*

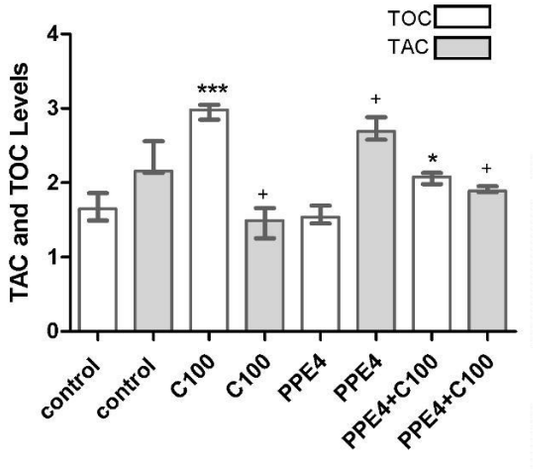

Figure 4. *The effects of PPE against cisplatin toxicity on neuronal oxidative system. TOC: total oxidant capacity (mmol/L); TAC: total antioxidant capacity (mmol/L); C100: 100 µM Cisplatin; PPE4: Punica granatum peel extract 400 mg/mL. The white columns show the TOC, the black columns show the TAC; error bars represent standard deviation; *p<0.05; ***p<0.001 and + p<0.05 versus control groups.*

3.4. Inflammation Findings

We investigated TNF-α mRNA expression level as a marker of inflammation in primary neuron cells. TNF-α expression levels; In our study, cisplatin group had significantly increased TNF-α expression levels compared to the control group. While there was no significant change in PPE4 group, it decreased the level of TNF-α increased by cisplatin in C100+PPE4 group (Figure 4c).

3.5. Apoptosis Findings

CAS 3 expression levels: In our study, CAS 3 expression levels increased significantly in cisplatin group compared to the control group ($p<0.001$). Although there was no significant change in PPE4 group, it decreased the level of CAS 3 increased by cisplatin in C100+PPE4 group (Figure 4a).

CAS 9 expression levels: In our study, CAS 3 expression levels increased significantly in cisplatin group compared to the control group ($p<0.01$). PPE4; CAS 9 level significantly decreased. It also increased the level of CAS 9 with cisplatin to the control level (Figure 4b).

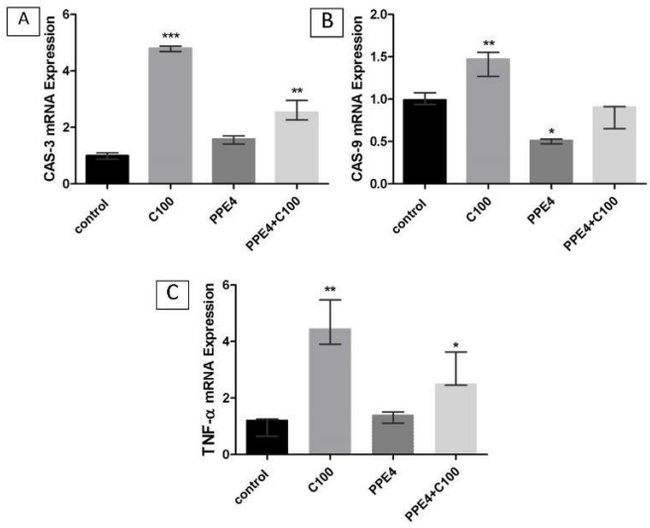

Figure 5. *Effects of PPE against cisplatin toxicity on neuron cells (A), CAS-3 mRNA expression (B), CAS-9 mRNA expression (C), TNF-α mRNA expression levels. The relative expression levels was calculated by the $2^{-\Delta\Delta Ct}$ method. C100: 100 µM Cisplatin; PPE4: Punica granatum peel extract 400 mg/mL; error bars represent standard deviation; *p<0.05; **p<0.01; versus control groups.*

4. DISCUSSION

Some studies have shown the effectiveness of different types of polyphenols using animal models of neurodegeneration to weaken or prevent neuronal death (16, 17). These compounds are antioxidant and anti-inflammatory properties and therapeutic effects due to the pharmacological effects including wide, are considered candidates for clinical trials in neurodegenerative disorders.

Pomegranate possesses a wide range of compounds, including polyphenols, alkaloids, and vitamins with potent free radical scavenging properties (8, 9). Today, *Punica granatum* is one of the most important antioxidant substance (11, 18). Antidiarrheic (19), antifungal (20), anti-ulcer (21), antibacterial (22), antitumoral and anticancer (23) effects have been shown in studies with *Punica granatum*. Oxidative stress has been known as imbalance between the free radicals and antioxidant defense system. Neurons are more sensitive to oxidative stress because of low activity of antioxidant enzymes (24). Some experimental studies support that reactive oxygen species were related with cisplatin cytotoxicity (5, 6).

As a result of stimulation of the expression of antiapoptotic genes in cancer cells, the development of chemoresistance is characterized by cisplatin administration (25). To eliminate this resistance, cisplatin should be administered in high doses, but it causes the most organ / tissue toxicity such as high doses of cisplatin, nephrotoxicity, hepatotoxicity and neurotoxicity

MTT assay was using evaluated cytotoxic effect and/or cell viability. According to the MTT assay, the cytotoxic effects of cisplatin was demonstrated by their strong inhibition on cell viability on neuron cells. One study showed the ability of cisplatin to penetrate into the brain where it inhibits neuronal stem cell proliferation (26). PPE3 and PPE4 significantly reduced toxicity caused by cisplatin ($p<0.001$) and increase the viability. Likewise, it was reported that cisplatin caused significant neurotoxicity via induction of lipid peroxidation and reduction in the potency of the antioxidant defense system (27).

In the present study, we investigated protective effect of PPE against cisplatin-induced oxidative effects by determining TAC and TOC levels. Cisplatin application decreased TAC level and TOC level was increased by Cetin et al. (12). In our current study, TAC level was significantly decreased with C100 application and a significant increase was observed in TOC level. However, in the PPE4 + C100 groups, the TAC level increased significantly compared to the C100 group, and the level TOC decreased significantly. In short, PPE4 reduced the oxidative damage due to its antioxidant effect induced by cisplatin (Figure 3). Changes in TNF-α mRNA expression levels were observed with real time PCR to demonstrate the antioxidant activity of PPE4. Parallel to TAC and TOC ELISA result; compared with the healthy group, the TNF-α mRNA expression level was increased by cisplatin administration, whereas the PPE4 normalized TNF-α mRNA expression levels.

Excessive accumulation of intracellular ROS has been recognized as the strongest trigger of cisplatin-induced initiation of CAS 3 and 9 activities, resulting in increased apoptosis (28). Marullo et al. showed that cells lacking functional mitochondria were more resistant to cisplatin-induced cell death (29). We have reached similar results. The results showed that PPE4 is able to protect primary neuron cells through inhibition of apoptosis, as proved by the attenuation of mRNA expression of proapoptotic gene CAS 3 and CAS 9 activation (Figure 3a).

5. CONCLUSION

Our in vitro experience: PPE4, provided protection against the neurotoxic effects of cisplatin in rat primary neurons. The protective mechanisms may be related to antioxidant activities and botanical phenolic components of PPE. Hence, inclusion of PPE in cisplatin-based chemoradiotherapy can be an effective strategy to counter cisplatin-induced neurotoxicity. Further research is needed to explain the exact mechanism of the potential neuroprotective effects of PPE.

Ethics Committee Approval: This study was conducted with permission from the Local Ethics Committee of Kafkas University Animal Experiments dated 25.01.2016 numbered 2016–016. A total of 10 newborn Spraque Dawley rats obtained from the experimental animal laboratory were used.

Conflict of interest: Part of the Study presented orally at "7th drug chemistry conference: Design, synthesis, production and standardization of drug active substances."

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