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Effect of Gallic Acid on PTZ-induced Neurotoxıcıty, Oxidative Stress and Inflammation in SH-SY5Y Neuroblastoma Cells

SH-SY5Y Nöroblastoma Hücrelerinde PTZ ile Oluşturulan Nörotoksisite, Oksidatif Stres ve İnflamasyon Üzerine Gallik Asidin Etkisi

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ABSTRACT

Aim: Human neuroblastoma cell lines are widely used to elucidate the cellular and molecular mechanisms of neurotoxicants and to facilitate the prioritization of in vivo testing. Pentylenetetrazole (PTZ) is a tetrazole derivative. Although PTZ is the most commonly used chemical to create an in vivo and in vitro epilepsy (EP) model, its mechanism of action in neuronal cells has not been fully elucidated. Gallic acid (GA) has broad biological properties such as antioxidant, anti-microbial, and anti-inflammatory activities. This study aimed to investigate the effect of GA on PTZ-induced neurotoxicity in neuroblastoma cells.

Methods: For the study, four groups were formed from SH-SY5Y neuroblastoma cells as control (C), GA (100 µM), PTZ (30 µM), and PTZ+GA. In the study, total antioxidant and oxidant status (TAS and TOS), inflammatory cytokines (TNF α, IL 1β, and IL 6), lipid peroxidation levels as malondialdehyde (MDA), glutathione peroxidase (GSHPx), and glutathione (GSH) levels in the SH-SY5Y neuroblastoma cells were determined.

Results: The results showed that PTZ treatment caused neurotoxicity in the neuroblastoma cell line and increased TOS, TNF α, IL 1β, IL 6, and MDA levels while decreasing TAS, GSH, and GSHPx levels. This situation improved with GA treatment.

Conclusion: As a result, it was determined that GA treatment showed a protective effect in the PTZ-induced neural toxicity model in SH-SY5Y human neuroblastoma cell lines.

Keywords: Neurotoxicity, SH-SY5Y neuroblastoma cell, Gallic acid, Pentylenetetrazole

ÖZ

Amaç: İnsan nöroblastoma hücre hatları, nörotoksik maddelerin hücresel ve moleküler mekanizmalarını aydınlatmak ve in vivo testlerin önceliklendirilmesini kolaylaştırmak için yaygın olarak kullanılmaktadır. Pentilenetetrazol (PTZ) bir tetrazol türevidir. PTZ, in vivo ve in vitro epilepsi (EP) modeli oluşturmak için en yaygın kullanılan kimyasal olmasına rağmen, nöronal hücrelerdeki etki mekanizması tam olarak aydınlatılamamıştır. Gallik asit (GA) antioksidan, anti-mikrobiyal ve anti-enflamatuar aktiviteler gibi geniş biyolojik özelliklere sahiptir. Bu çalışma, GA'nın nöroblastoma hücrelerinde PTZ kaynaklı nörotoksisite üzerindeki etkisini araştırmayı amaçlamıştır.

Yöntem: Çalışma için SH-SY5Y nöroblastoma hücrelerinden kontrol (K), GA (100 µM), PTZ (30 µM) ve PTZ+GA olmak üzere dört grup oluşturulmuştur. Çalışmada SH-SY5Y nöroblastoma hücrelerinde toplam antioksidan ve oksidan durumu (TAS ve TOS), inflamatuvar sitokinler (TNF α, IL 1β ve IL 6), glutatyon peroksidaz (GSHPx), glutatyon (GSH) seviyeleri ve malondialdehit (MDA) olarak lipid peroksidasyon seviyeleri belirlenmiştir.

Bulgular: Sonuçlar PTZ tedavisinin nöroblastoma hücre hattında nörotoksisiteye neden olduğunu ve TOS, TNF α, IL 1β, IL 6 ve MDA seviyelerini artırırken TAS, GSH ve GSHPx seviyelerini azalttığını göstermiştir. Bu durum GA tedavisi ile düzelmiştir.

Sonuç: Sonuç olarak, GA tedavisinin SH-SY5Y insan nöroblastom hücre hatlarında PTZ kaynaklı nöral toksisite modelinde koruyucu bir etki gösterdiği belirlenmiştir.

Anahtar Kelimeler: Nörotoksisite, SH-SY5Y nöroblastom hücresi, Gallik asit, Pentilenetetrazol

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Introduction

Epilepsy (EP), often characterized by recurrent epileptic seizures, consists of various neurological conditions [1]. Although many antiepileptic drugs are used in the clinic to treat EP, approximately 30% of patients still cannot control their seizures medically [2]. Withal, many studies have shown that recurrent and prolonged seizures often lead to neuronal damage [3]. Despite the high prevalence and extensive research on EP, a common neurological disorder, the cellular and molecular mechanisms underlying epileptogenesis and neuronal injury remain unclear. The human SH-SY5Y neuroblastoma cell lines are widely used to elucidate neurotoxic substances' cellular and molecular mechanisms and to prioritize in vivo testing [1,4]. Epileptic activity in vivo and in vitro experimental models is mainly induced by the local administration of epileptogenic drugs, including the widely used pentylenetetrazole (PTZ) [5,6].

PTZ, a bicyclic tetrazole derivative, is a GABA-A (γ-aminobutyric acid type A) receptor antagonist and causes excitation in neurons [7]. Due to its stimulating effect, PTZ is the most widely used chemical to create an in vivo and in vitro EP model [1,3,5,6]. Although neurobiological processes and structural changes resulting from stimulation with PTZ have been investigated, the mechanisms that trigger neurotoxicity have not been fully elucidated. Extensive experimental studies have shown that inflammation and oxidative stress are crucial in developing neurological diseases [1,8,9]. Strengthening neuronal cell defense against oxidative stress and inflammatory processes with exogenous antioxidant-anti-inflammatory substances can provide adequate protection [10]. Therefore, improving antioxidant-anti-inflammatory compounds from natural sources is of great scientific interest.

Gallic acid (GA) has broad biological properties such as antioxidant, anti-microbial, and anti-inflammatory activities [11]. Some recent studies have demonstrated the practical activity of phytoconstituents such as GA in preventing neuronal damage (3,4,5-trihydroxy benzoic acid) [11]. GA is a flavonoid found in natural foods such as sumac, green tea, witch hazel, and oak bark and has critical biological effects [12,13]. Recently, interest in GA has increased due to its different effects, such as anticancer, anti-inflammatory, and antioxidant [13-15]. However, more research must be done on its neuroprotective effect [8,10,13]. In addition, the mechanism of its protective effect on nerve cells has yet to be understood.

PTZ treatment has been reported to cause neuronal damage in experimental in vitro [1,3] and in vivo [16,17]. However, the mechanisms by which PTZ triggers cell damage in EP models remain unclear. Inflammation and oxidative stress often play a role in the pathology of secondary neuronal damage following EP [18,19]. Experimental studies suggest that GA reverses oxidative stress and suppresses inflammation [8,10]. However, the roles and mechanisms of action of GA on PTZ-induced neuronal damage are not yet known. Although there are limited studies investigating PTZ-induced neural toxicity in human neuroblastoma cells, there is no research examining the regulatory role of GA in this process, which makes this study original.

This study aimed to investigate the effect of PTZ, widely used in the experimental neural toxicity model, on human neuroblastoma cells and the curative effect of GA.

Materials and Methods

Chemicals and ELISA Kits

Gallic acid (Cat; G7384) was obtained from Sigma Aldrich C. (St Louis, USA). Pentylenetetrazole (Cat; P6500) was purchased by Sigma Aldrich C. (St Louis/USA). IL 6 (Cat; KET6017), IL 1β (Cat; KET6013), and TNF α (Cat: KET6032) were purchased from Abbkine/Scientific C. (Wuhan, China). SH-SY5Y cells (Cat; CRL-2266) were purchased from ATCC (VA, USA). Total Oxidant and Total Antioxidant Capacity (TOS product no: RL0024 and TAS product no: RL0017) ELISA kit was obtained from Rel Assay (Gaziantep/Türkiye). Other chemicals used in glutathione, glutathione peroxidase, and lipid peroxidation analyses were purchased from Sigma Chemical Inc. (St. Louis, USA).

Cell Culture and Experimental Groups

A growth medium was prepared for the cells

used in the research according to the instructions provided by the seller. FBS (Cat; SV30160.03, cytiva) (10 %) and penicillin/ streptomycin (Cat; LM-A4118, biosera) (1 %) were added to equivalent volumes of Ham's F12 (Cat; L0135, biowest) and DMEM (Cat; L0064, biowest) as growth medium contents. Cells (6-8 passages) previously purchased from ATCC and stored in a nitrogen tank were used. The cells taken from the nitrogen tank were passaged, and after the cells reached 80-85% confluence, they were divided into four groups, and this process was repeated. Cells were cultured in 25 cm2 culture flasks in an incubator at 37 °C under a 5 % CO_2 atmosphere. Human SH-SY5Y neuroblastoma cells were divided into four groups and incubated according to the experimental procedure. PTZ and GA were freshly prepared on the experimental days.

Control (C) group (n=5), no treatment was applied to the control group.

GA group (n=5), Cells were added 100 µM GA and incubated for 24 hours [20].

PTZ group (n=5), cells were added 30 µM PTZ and incubated for 24 hours [1].

PTZ+GA group (n=5), the cells were incubated by PTZ (30 µM) and GA (100 µM) for 24 hours.

After the incubation period was completed, the cells were washed with fresh 1xPhosphate Buffered Saline (PBS, Biochrom/Germany), and 0.25 % Trypsin-EDTA (Sigma-Aldrich) was applied to separate the cells from the flask floor. After completing the experimental steps, analyses were performed for all groups.

Preparation of Cells Homogenates

For each group, cells were transferred into separate sterile falcon tubes and centrifuged according to the kit procedure (1000 rpm and 20 min). After centrifugation, the supernatants on the top of the falcon tubes were removed with the help of an automatic pipette, the cells were suspended in PBS, and a cell suspension with a density of approximately 1×10^6 cells/ml was obtained. The cell structure was lysed (PBS) by freeze-thaw repetition, and the mixture was centrifuged at 4000 rpm for 10 minutes at 4 °C after removing the cytoplasmic components. The supernatant remaining at the top of the falcon tubes was removed with pipettes and taken in Eppendorf tubes for analysis. The Bradford protein assay kit (Merck-Millipore) measured total protein levels in the groups.

Analyses

Measurement of Total Oxidant-Antioxidant and Inflammatory Cytokines Levels in the SH-SY5Y Neuroblastoma Cells

PTZ-induced toxicity induction of inflammatory cytokines (TNF α, IL 1β, and IL 6) ELISA kits determined levels in the supernatants of the SH-SY5Y cell line. For the analyses, supernatants were first incubated at 37 °C for 60 minutes by the protocols specified by the companies for commercial kits and then placed in 96-well plates with automatic pipettes. The supernatant and standard samples placed on the plate were incubated for 60 minutes, followed by washing steps, and then staining solutions were added and incubated for 15 minutes. A stop solution was added at the end of all these procedures, and absorbance values were read at 450 nm on an ELISA (BioTek ELx808™) microplate reader [21].

PTZ-induced toxicity induction of Total antioxidant status (TAS) and Total oxidant status (TOS) ELISA kits determined levels in the supernatants of the SH-SY5Y cell line. For TAS analysis, cell culture supernatants were used, and culture samples, kit standard (mmol Trolox eq/L), and dH_20 were mixed with Reagent 1 (Buffer, 200 µL) in 96-well plates and incubated for 5 min according to the manufacturer's protocols. The absorbance was measured at 660 nm (the first absorbance of the sample). Then, Reagent 2 (Color ABTS Radical Solution, 30 µL) was added, and mixtures were incubated at 37 °C for 5 min; an ELISA microplate reader monitored absorbance at 660 nm (second absorbance of the sample). Each sample data was calculated using the kit's standard (equivalent to 1 mmol/L of Trolox). For TOS analysis, cell culture supernatants were used. For the dilution step, 5 µL of Standard 2 and 1 mL of distilled water were transferred to Eppendorf and vortexed. Then, 5 µL of this solution was transferred to Eppendorf, and 20 mM H_2O_2 was prepared by adding 1 mL of water. For TOS analysis, Reagent 1 (Assay buffer, 200 µL) was added to each well, and absorbances

were measured at 530 nm (first absorbance of the sample). Next, Reagent 2 (Prochromogen solution, 10 µL) was added, and samples were incubated for 5 min at 37 °C. Finally, absorbance was monitored at 530 nm using an ELISA microplate reader (second absorbance of the sample). The assay was calibrated with hydrogen peroxide, and the results are expressed in micromolar hydrogen peroxide equivalents per litre (µmol H_2O_2 equivalents/L). The percentage ratio of the TOS to the TAS was accepted as the oxidative stress index (OSI), an indicator of the degree of oxidative stress. For calculations, the resulting unit of TAS, mmol Trolox eq/L, was converted to µmol Trolox eq/L, and the OSI value was calculated using the following formula: OSI = [TOS (µM H_2O_2 eq/L) / TAS (µmol Trolox eq /L)] × 100.

Measurement of Lipid Peroxidation, Glutathione, and Glutathione Peroxidase levels in the SH-SY5Y Neuroblastoma Cell

Lipid peroxidation activity, which is known as malondialdehyde (MDA) release in PTZ-induced nephrotoxicity in SH-SY5Y human neuroblastoma cells, was determined by thiobarbituric acid (TBARS) reaction in a highly sensitive spectrophotometer (V-730 UV-Visible Spectrophotometer, Japan) according to the method of Placer et al. All cell groups were reconstituted with 1/9 (2.25 ml) TBARS solution. The experiment used a mixture of 0.25 ml phosphate buffer and 1/9 of TBARS as a blind. Samples and blind were kept in 100 °C water for 20 minutes [22,23]. It was then cooled on ice and centrifuged at 1000 g for 5 min. The upper pink liquid was taken with an automatic pipette and read against the blind in a spectrophotometer at 532 nm wavelength in a 1 cm light transmission cuvette. The standard was standard: 1, 1, 1, 3, 3 tetraethoxy propane solution prepared in the same proportions. Values were determined as µmol/g protein.

Glutathione (GSH) levels of SH-SY5Y human neuroblastoma cells were determined spectrophotometrically at 412 nm using the Sedlak and Lindsay method [24]. The cells (10^6 cells per mL) were transferred to sterile falcon tubes with the help of an automatic pipette and centrifuged to separate the proteins after mixing with 10% trichloroacetic acid. After centrifugation, 0.1 ml of the supernatant remaining on the falcon tube was taken and placed in a glass tube, 0.5 mL 5.5-dithiobis (2-nitrobenzoic acid), 2 mL phosphate buffer (pH 8.4), and 0.4 mL distilled water were added. The resulting sample was read at 412 nm in a spectrophotometer. Values were determined as µmol/g protein.

Glutathione peroxidase (GSHPx) levels of SH-SY5Y human neuroblastoma cells were determined spectrophotometrically at 412 nm by Lawrence and Burk's method [25]. The activity of GSHPx was expressed as international units (IU) of oxidized glutathione/g protein. Total protein in cells was assessed spectrophotometrically at 595 nm using Bradford reagent.

Statistical Analysis

All data were expressed as mean ± standard deviation (SD). Data analyses were performed with the SPSS (version 17.0, software, USA) program. One-way ANOVA was used to evaluate the differences between the groups. Post-hoc Tukey test was used in all data with a statistically significant difference. $p \leq 0.05$ was considered statistically significant.

Results

Effect of Gallic Acid on TOS and TAS Levels in SH-SY5Y Neuroblastoma Cells

This study measured changes in TOS and TAS levels between experimental groups with commercial Elisa kits. Figure 1 shows that GA treatment modulates the decrease in TAS levels (Figure 1B) and the increase in TOS levels (Figure 1A) in PTZ-induced SH-SY5Y cells. A significant increase in TOS and OSI levels (Figure 1A and 1C) was observed in the PTZ-treated group was compared to C, GA, and PTZ+GA groups ($p \leq 0.05$), and in parallel, a significant decrease in TAS levels (Figure 1B) was observed in the PTZ-treated group was compared to C, GA and PTZ+GA groups ($p \leq 0.05$). The decrease in TAS levels and increase in TOS levels were regulated by GA treatment.

Effect of Gallic Acid on Inflammatory Cytokines Levels in SH-SY5Y Neuroblastoma Cells After PTZ-induced Cytotoxicity

The changes in inflammatory cytokines (TNF α, IL 1β, and IL 6) levels in cells against PTZ-induced cytotoxicity of gallic acid in the groups formed were measured with the Elisa kits. GA treatment modulated PTZ-induced inflammation (TNF α, IL 1β, and IL 6) levels in the SH-SY5Y neuroblastoma cells are shown in Figure 2. When the PTZ-induced treated group was compared to the C, GA, and PTZ+GA groups between the groups, it was observed that the IL 1β, IL 6, and TNF α levels (Figure 2A, 2B, and 2C) increased considerably ($p \leq 0.05$). This situation was regulated by GA treatment.

The Gallic Acid Treatment Attenuated the PTZ-induced Changes in GSH, GSHPx, and MDA Levels.

The changes in GSH, GSHPx, and MDA levels in cells against PTZ-induced cytotoxicity of GA in the groups formed were measured spectrophotometrically. GA treatment modulated PTZ-induced lipid peroxidation and impaired antioxidant balance levels in the SH-SY5Y neuroblastoma cells are shown in Figure 3. When the PTZ-induced treated group was compared to the C, GA, and PTZ+GA groups between groups, it was observed that the GSH level (Figure 3A) decreased considerably ($p \leq 0.05$). GSHPx levels (Figure 3B) were significantly reduced between the groups when the PTZ-induced treated group was compared to the C, GA, and PTZ+GA groups ($p \leq 0.05$). MDA levels (Figure 3C) were significantly increased between the groups when the PTZ-induced treated group was compared to the C, GA, and PTZ+GA groups ($p \leq 0.05$). This situation was regulated by GA treatment.

Discussion

Abnormal electrical discharges in the brain characterize EP [1]. Generally, epileptiform activity is induced by local administration of epileptogenic drugs such as PTZ, widely used in both in vitro and in vivo experimental models [3,6]. Numerous experimental and clinical studies report that epileptic seizures cause neuron damage [16,26]. However, the mechanisms by which PTZ induces cell damage are unclear, so experimental models of PTZ-induced EP are attractive. It has been shown in some animal studies that oxidative stress has an essential role in the etiology of EP [1,16]. In the rat EP model created with PTZ, MDA levels increased in the hippocampus, while SOD and GSHPx levels decreased [16]. A similar study observed increased MDA levels in rats induced by PTZ, while a significant decrease was observed in CAT, SOD, and GSH levels [17]. An increase in lipid peroxidation, neutrophil infiltration, and oxidative stress parameters was reported in another PTZ-induced rat brain injury and memory impairment model [9,18]. As we highlighted, many studies emphasize that PTZ application causes neuronal damage in cell culture models performed in vitro [1,3,5] or animal models performed in vivo [16,17]. This study investigated how inflammation and oxidative stress markers changed in human SH-SY5Y neuroblastoma cells after PTZ administration. It was found that the levels of TOS and apoptosis were increased in PTZ-induced human SH-SY5Y neuroblastoma cells, whereas the levels of TAS were significantly decreased [27].

Our study observed that the TOS level increased considerably in the PTZ group compared to the C group, whereas the TAS level decreased significantly. Moreover, in our research, while MDA levels increased in the PTZ group, GSH and GSHPx levels decreased. In our study, which aimed to determine the mechanisms that trigger neurotoxicity due to stimulation with PTZ, we also examined the parameters of inflammation. We determined that inflammatory cytokines (IL 1β, TNF α, and IL 6) levels were significantly increased in the PTZ group compared to the other groups. Thus, we demonstrated significant increases in inflammatory cytokine levels in a PTZ-induced in vitro model of EP. Consistent with our results, Khatoon et al. reported that TNF α and IL 6 levels in the cortex and hippocampus of mice induced by PTZ were significantly increased compared to the C group [28]. In addition, in the EP model created in HT-22 cells, an increase in inflammatory cytokines (TNF α, IL 1β, and IL 6) levels was observed in the cells in the PTZ group [6]. Similarly, Ahlatçı et al. showed a significant increase in inflammatory cytokines (TNF α, IL 1β, and IL 6) levels in PTZ-induced cells [1].

There are limited studies on the neuroprotective effect of GA, which has essential effects such as anticancer, anti-inflammatory, and antioxidant

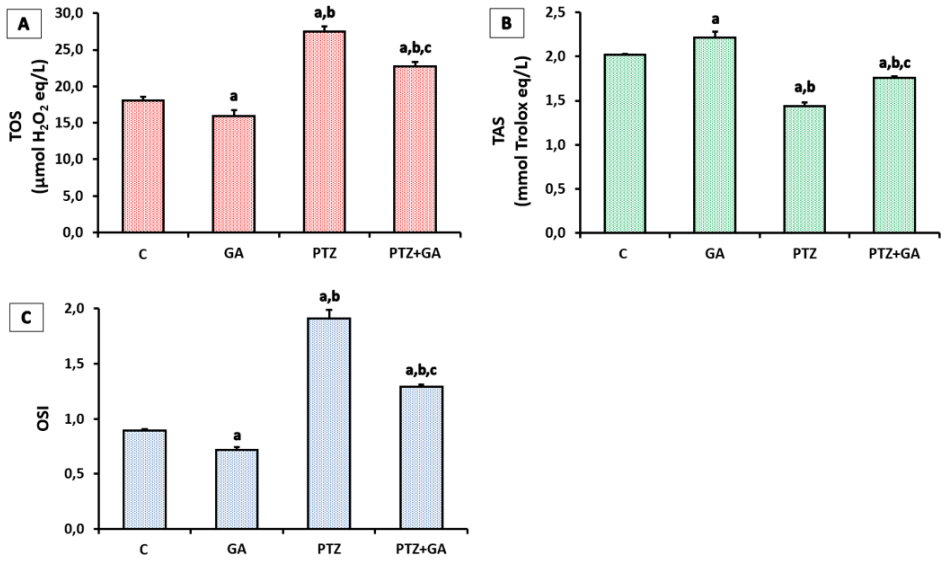

Figure 1. The effect of PTZ and GA treatment on TOS (A), TAS (B), and OSI (C) levels (mean ± SD). (ap≤ 0.05 vs. C group, bp≤ 0.05 vs. GA group, cp≤ 0.05 vs PTZ group).

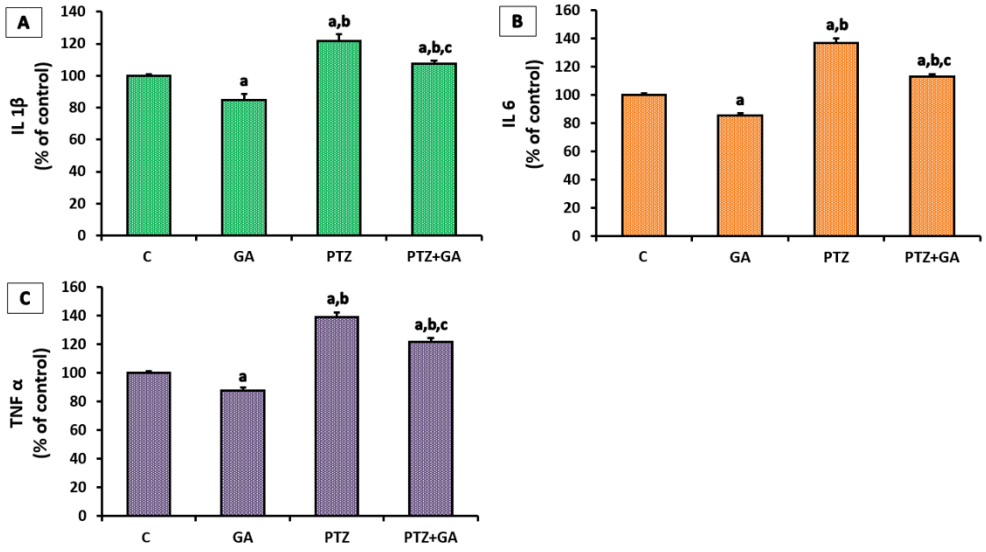

Figure 2. The effect of PTZ and GA treatment on IL 1β (A), IL 6 (B), and TNF α (C) levels (mean ± SD). (ap≤ 0.05 vs. C group, bp≤ 0.05 vs. GA group, cp≤ 0.05 vs PTZ group).

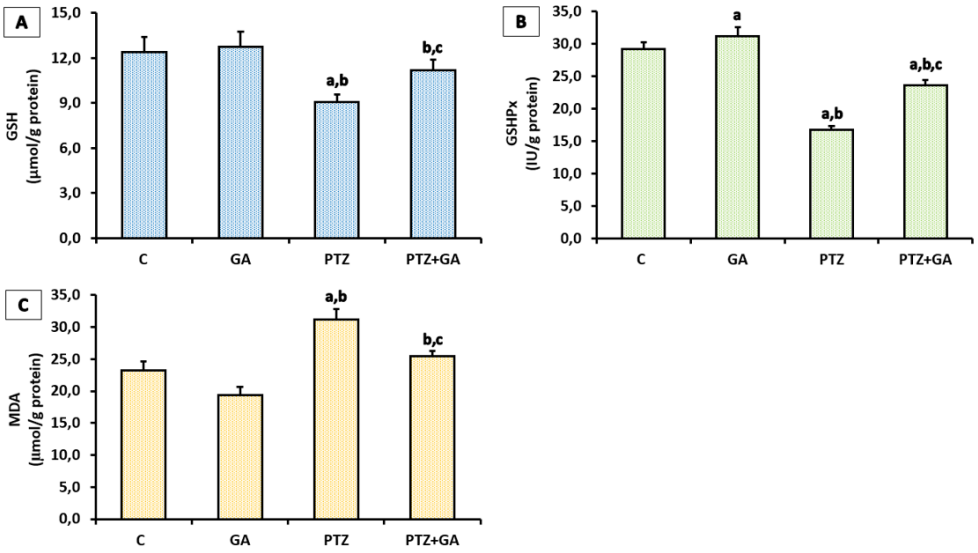

Figure 3. The effect of PTZ and GA treatment on GSH (A), GSHPx (B), and MDA (C) levels (mean ± SD). (ap≤ 0.05 vs. C group, bp≤ 0.05 vs. GA group, cp≤ 0.05 vs PTZ group).

[15,29,30]. In this study, we investigated the possible mechanism of the protective effect of GA on nerve cells in an in vivo experimental EP model. For this purpose, we administered GA treatment to groups with and without PTZ. Our study observed that GA incubation with PTZ reduced oxidative stress and suppressed inflammation. We showed that the use of GA in the GA group increased the GSHPx and GSH levels and decreased the MDA level compared to the PTZ group. Moreover, in line with the literature, inflammatory cytokines (TNF α, IL 1β, and IL 6) levels decreased in the same group [31,32]. Like our results, Maurya et al. determined that GA treatment significantly reduced lung, liver, kidney, and spleen MDA levels and showed significant improvement in SOD activity in septic mice [30]. Similarly, inflammatory cytokines (TNF α, IL 1β, and IL 6) levels decreased in the same group. We also found a significant improvement in MDA, GSH, and GSHPx levels in the PTZ+GA group compared to the C group. However, there was a significant improvement in oxidative parameters compared to the C group. Similarly, there was a significant improvement in inflammatory cytokine (TNF α, IL 1β, and IL 6) levels compared to the C group. This study showed that GA improved PTZ-induced oxidative stress and inflammation. Our study proved that GA exerts neuroprotective effects through antioxidant and anti-inflammatory mechanisms in SH-SY5Y cells in an in vitro EP model.

Lımıtatıons: This study used only the SH-SY5Y human neuroblastoma cell line to model EP with PTZ. In addition, studies investigating signaling pathways at the molecular level are needed to understand better how PTZ affects molecular mechanisms.

Conclusion: There is current research showing that EP attacks cause neurotoxicity. Since attacks cannot be prevented entirely in many patients, minimizing the neuronal damage caused by attacks would be a correct additional treatment approach for EP. In this context, it is vital to determine the mechanisms underlying neuronal damage and find treatments that will reduce neuronal damage.

This study showed that in an in vitro EP model, PTZ induced neurotoxicity in SH-SY5Y neuroblastoma cells by disrupting the oxidant/antioxidant balance and increasing the release of inflammatory cytokines. In addition, the regulatory effectiveness of GA was determined, and essential preclinical data was provided to the literature. Although these data show the possibility of using natural substances such as GA to reduce the damage that may occur during attacks in EP patients, further preclinical research is needed.

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Ethics Declarations: The current study has no study with human and human participants. The study is not subject to ethics committee approval.

AORCID and Author Contribution: **YY (0000-0002-5613-6906)** was responsible for formulating the hypothesis, analyses and writing the report.

Abbreviations

DMEM, Dulbecco's Modified Eagle Medium

FBS, Fetal Bovine Serum

GA, Gallic acid

GSHPx, Glutathione peroxidase

GSH, Glutathione

MDA, Malondialdehid

ROS, Reactive oxygen species

SH-SY5Y, Human neuroblastoma cell line

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