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NEUROPROTECTIVE EFFECT OF CHLORZOXAZONE AGAINST GLUTAMATE TOXICITY IN RAT PRIMARY CORTEX NEURON CULTURE
SIÇAN PRİMER KORTEKS NÖRON KÜLTÜRÜNDE GLUTAMAT TOKSİSİTESİNE KARŞI KLORZOKSAZONUN NÖROPROTEKTİF ETKİSİ

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ABSTRACT

Glutamate (Glut) toxicity is one of the main causes of neurological diseases. Chlorzoxazone (CZ) is a muscle relaxant used to decrease pain and inflammation associated with acute and chronic twists and bruises. Here, we objected to research the neuroprotective effect of CZ applied to reverse Glut-induced neurodegeneration in the neonatal cerebral cortex through anti-inflammatory and antioxidant mechanisms. Neonatal cortical neurons were exposed to Glut and different doses of CZ (10, 20, and 40 µM) were applied to assess the effect of CZ on Glut toxicity. We then examined changes in cell viability, inflammation, and oxidative stress. Our cell viability analysis showed that CZ protected cells from Glut-induced neuronal damage. In addition, the neuroprotective properties of CZ were evaluated by examining oxidative and antioxidant parameters such as MDA, MPO, CAT, GSH, GPx, and SOD. In line with the data obtained, it was observed that the cell viability rate decreased to 60% in the Glut group. However, with CZ application, the most significant increase in cell viability was seen at the 40 µM dose (86%), while the least increase was seen at 10 µM CZ (77%). It also proved that CZ increased the activity of antioxidant parameters while reducing oxidative parameters and inflammation. Therefore, the present findings collectively demonstrated that CZ potently inhibits Glut-induced injury in neonatal cortical neurons. The present work is the initial to show the protective effect of CZ in neonatal cortical neurons exposed to Glut excitotoxicity and suggesting that CZ may be used as a therapeutic agent.

Keywords: Chlorzoxazone, cortex, glutamate, neuron

ÖZ

Glutamat (Glut) toksisitesi, nörolojik hastalıklara zemin hazırlayan ana sebeplerden biridir. Klorzoksazon (CZ), akut ve kronik morluklar ve burkulmalarla ilişkili ağrı ve iltihabı azaltmak için kullanılan kas gevşeticidir. Burada, yenidoğan serebral kortekste Glut'un neden olduğu nörodejenerasyonu tersine çevirmek için uygulanan CZ'nin nöroprotektif etkisini anti-inflamatuar ve antioksidan mekanizmalar yoluyla araştırmayı amaçladık. Yenidoğan kortikal nöronları Glut'a maruz bırakıldı ve CZ'nin Glut toksisitesi üzerindeki etkisini değerlendirmek için CZ çeşitli dozlarda (10, 20 ve 40 µM) uygulandı. Ardından hücre canlılığı, oksidatif stres ve inflamasyondaki değişiklikleri inceledik. Hücre canlılık analizimiz, CZ'nin Glut kaynaklı nöranal hasardan hücreleri koruduğunu göstermiştir. Ayrıca CZ'nin nöroprotektif özelliği MDA, MPO, CAT, GSH, GPx ve SOD gibi oksidatif ve antioksidan parametrelerin incelenmesi ile değerlendirildi. Elde edilen veriler doğrultusunda,hücre canlılık oranı Glut grubunda % 60'a kadar düştüğü gözlendi. Ancak CZ uygulaması ile birlikte hücre canlılığında en anlamlı artış 40 µM dozunda (%86) görülürken, en az artış 10 µM CZ (%77) görüldü. Ayrıca CZ'nin oksidatif parametreleri ve inflamasyonu azaltırken, antioksidan parametrelerin aktivitesini arttırdığını kanıtladı. Bu nedenle mevcut bulgular toplu olarak CZ'nin Glut kaynaklı hasarı yenidoğan kortikal nöronlarında güçlü bir şekilde önlediğini göstermiştir. Mevcut çalışma, Glut eksitotoksisitesine maruz kalan yenidoğan korteks nöronlarında CZ'nin koruyucu etkisini gösteren ilk çalışmadır ve CZ'nin terapötik bir ajan olarak kullanılabileceğini göstermektedir.

Anahtar kelimeler: Klorzoksazon, korteks, glutamat, nöron

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INTRODUCTION

The brain is one of the largest and most complex organs in the body; It consists of billions of neurons that network with countless connections and synapses. The neurons that make up the cerebral cortex coordinate higher-level processes such as consciousness, thought, and emotion.[1] Glutamate (Glut), which is an excitatory neurotransmitter[2] in the cerebral cortex, is important in the plasticity and development of synaptic connections.[3] In certain neurological diseases, such as Parkinson's disease, cerebral hypoxia/anoxia, multiple sclerosis, or Alzheimer's disease, extracellular Glut levels increase causing irreversible neuronal damage,[4] which in turn causes calcium (Ca^{2+}) accumulation in the intracellular matrix[5] and N-methyl-D-aspartate (NMDA) can cause increased flux through its receptors.[6] Thus, Ca^{2+} accumulating in the cell leads to oxidative stress[7], and activates the ischemic[8] and apoptotic cascades.[9] In addition, the NMDA receptors play an important role in the central sensitization processes associated with hyperalgesia.[10]

Free radicals, which are constantly produced in the cell, are destroyed by antioxidant defense systems produced during normal metabolism in the body. Antioxidants prevent or delay cell damage by scavenging free radicals within the cell. Antioxidants can be produced naturally in the body or obtained externally from food.[11] Antioxidant defenses have complex enzymatic and non-enzymatic systems. For this reason, the cell's first, second, and third antioxidant defense mechanisms are referred to. The first line of defense is the superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GSH-Px) antioxidant defense systems, which suppress the formation of free radicals.[12] SOD, CAT, GSH and GSH-Px enzyme activities form the basic defense system in the cell and play a key role in combating diseases caused by oxidative damage. The production of free radicals in the cell leads to the disruption of the neuronal glucose transporter Glut3, Glut transporters, Na+/K+ ATPase pump, membrane protein functions, ion transport, and kinase activity, causing neurodegenerative diseases.[13] Antioxidants prevent or reduce tissue damage by scavenging free radicals.

Chlorzoxazone (CZ) is currently used as a muscle relaxant. It acts centrally in the subcortical areas of the brain and mainly to the spinal cord by suppressing reflexes. Therefore, it is reported to have a sedative effect.[14,15] CZ is also present as a marker of cytochrome P4502E1 in rats and human brains.[16] CYP2E1 is involved in the metabolism of chemicals that affect the central nervous system, such as anesthetics, muscle relaxants, and ethanol.[17] Food and Drug Administration-approved CZ can lighten inflammatory infiltration to decrease inflammation and pain associated with acute and chronic twits and bruises.[18] This study objected to research on the potential neuroprotective effects of CZ against Glut toxicity in vitro.

MATERIALS AND METHODS

Primary Neuron Culture

CZ and Glut were acquired from Sigma-Aldrich (St. Louis, Missouri, USA). This study was conducted with the approval of the Ataturk University Animal Experiments Local Ethics Committee (27 October 2022 E-42190979-000-2200337716). Neonatal Sprague-Dawley rats, less than 24 hours old, were used to harvest cortical neurons. Cortices removed from decapitated pups were transferred to 5 mL of Hanks balanced salt solution, macrolysed with a scalpel, and then digested with 0.25% trypsin-ethylenediaminetetraacetic acid. 88% neurobasal medium, 10% fetal bovine serum, 2% B -27 supplement, and 0.1% antibiotics (penicillin-streptomycin and amphotericin B) (Thermo Fisher Scientific, USA) were added to the cells that were centrifuged at 1200 rpm for 5 minutes. The cell medium was incubated for 10 days (5% CO_2, 37°C) and changed every 3 days. To induce toxic injury, neurons except the control group were exposed to Glut at a concentration of 10^{-5}Mfor 5 minutes. [19] Then, to evaluate the role of CZ in Glut toxicity, different doses of CZ (10, 20, and 40 μM) were applied to separate wells and incubated for 24 hours.

Cell Viability Analysis

It was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis method, which is one of the enzymatic test methods widely used in the evaluation of proliferation and cytotoxicity. The method uses MTT, which is actively absorbed by living cells. The tetrazolium ring is catalyzed by mitochondrial succinate dehydrogenase and turns into blue-violet-colored insoluble formazan crystals, which are considered an indicator of the number of metabolically active cells. These crystals formed dissolve in dimethyl sulfoxide. Then, cell viability is determined spectrophotometrically, and the viability of untreated cells is accepted as 100%, and the viability of treated cells is determined as a percentage (%) compared to these cells.[20]

Cell viability was evaluated by the MTT, yellow tetrazole assay method. The potential cytotoxic effect of CZ on Glut toxic neuron cells was measured using the MTT kit according to the manufacturer's instructions (Sigma, Mo, USA). Stock solution (MTT) at 10% concentration was added to the 96-well plate. After incubation for 4 hours, 100 μL of dimethyl sulfoxide was added. Optical density was read at 570 nm (BioTek Instruments, USA).

Biochemical Analysis

Cell medium was gathered 1 day after toxicity administration and assayed according to the manufacturer's instructions.

Malondialdehit (MDA) levels MDA ELISA kit (E-EL-0060/MDA; Elabscience, USA) was used in the evaluation and the method was performed as in the instructions. OD was measured spectrophotometrically at 450 nm wavelengths. MDA activity was expressed as ng/mL.

SOD levels SOD ELISA kit (E-EL-R1424/SOD; Elabscience, USA) was used in the evaluation and the method was performed as in the instructions. Optical density (OD) was measured spectrophotometrically at 450nm wavelengths. SOD activity was expressed as ng/mL.

GSH levels GSH ELISA kit (E-EL-0026/GSH; Elabscience, USA) was used in the evaluation and the method was performed as in the instructions. OD was measured spectrophotometrically at 450nm wavelengths. GSH

activity was expressed as µg/mL.

CAT levels CAT ELISA kit (E-BC-K031-S/CAT; Elabscience, USA) was used in the evaluation and the method was performed as in the instructions. OD was measured spectrophotometrically at 405nm wavelengths. CAT activity was expressed as U/mL.

GSH-Px levels GPx ELISA kit (E-BC-K096-S/GSH-Px; Elabscience, USA) was used in the evaluation and the method was performed as in the instructions. OD was measured spectrophotometrically at 412 nm wavelengths. GSH-Px activity was expressed as µmol/L.

Myeloperoxidase (MPO) levels MDA ELISA kit (E-UNEL-H0048/MPO; Elabscience, USA) was used in the evaluation and the method was performed as in the instructions. OD was measured spectrophotometrically at 450 nm wavelengths. MDA activity was expressed as ng/mL.

Statistical Analysis

Statistics were performed using the post hoc Tukey test (IBM SPSS 20) (*p<.05*) and one-way analysis of variance. Data are presented as mean ± standard deviation.

RESULTS

Effect of CZ on Cell Viability

The cytotoxic effect of CZ was determined using the MTT method. The cytotoxic effect of CZ applied at various concentrations (10, 20, and 40 µM) on Glut toxicity established in primary cortical neuron culture is shown in Figure 1. In the evaluation of cell viability obtained at the end of the 24-hour incubation period of Glut toxicity-induced primary neuron cells applied to CZ, it was observed that the cell viability rate decreased to 60% following the application of 10^5 M Glut. It was determined that there was an increase in cell viability following CZ application. While the most significant increase was observed following 40 µM CZ application (86%), the least increase was observed following 10 µM CZ application (77%). While it was observed that the groups applied CZ at 20 µM concentration had a statistically significant protective effect on cell viability compared to the Glut control (*p<.05*), the most significant protective effect was detected in the group applied 40 µM CZ (*p<.001*) (Figure 1) (Table 1).

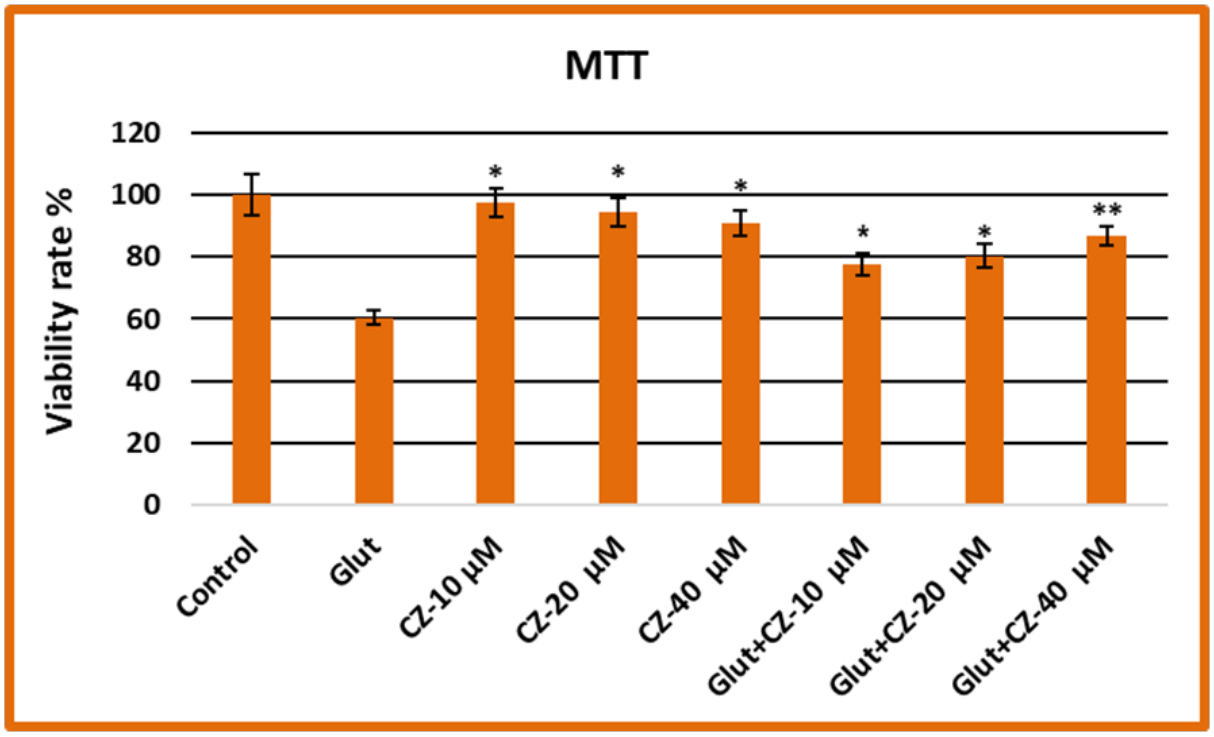

Figure 1: Effects of CZ on the cell viability. Data are expressed as the mean ± standard deviation. **p<.05* vs. Glut group, ***p<.001* vs. Glut group.

Table 1: Statistical results on the effect of CZ on cell viability.

Groups	Mean±SD	*p*
Control	100±13.04	0.060
Glut	60.46±2.5	
CZ-10 µM	97.57±10.01*	0.048
CZ-20 µM	92.39±10.93*	0.012
CZ-40 µM	88.83±9.24*	0.026
Glut+CZ-10 µM	77.02±5.33*	0.008
Glut+CZ-20 µM	81.24±7.50*	0.035

Data are expressed as the mean ± standard deviation.**p<.05* vs. Glut group, ***p<.001* vs. Glut group.

Effect of CZ on Glut-induced Oxidative Stress

Following the application of CZ at various concentrations (10, 20, and 40 µM) for 24 hours on Glut toxicity in primary neuron culture, cell culture medium was taken and antioxidant and oxidant capacities were measured with the help of a commercial kit (Figure 2) (Table 2). MPO and MDA results show the oxidant and free radical levels in the cell culture medium. The findings showed that the Glut group had the highest oxidant capacity, which caused the intracellular stress factor to induce toxicity and increased cell death. Consistent with the GSH, GSH-Px, CAT, and SOD results, a statistically significant decrease was detected in the MPO and MDA levels of the CZ groups (10, 20, and 40 µM) (*p<.001*).

DISCUSSION

We show that CZ protects cortex neurons against Glut-induced cell death. The current study provides strong evidence that CZ has a neuroprotective effect on Glut-induced excitotoxicity by suppressing oxidative stress.

Glut functions as an excitatory neurotransmitter in the cerebral cortex.[2] In case of excessive exposure to Glut, it is the main promoter of neuronal damage in pathological diseases associated with the central nervous system due to the activation of oxidative stress

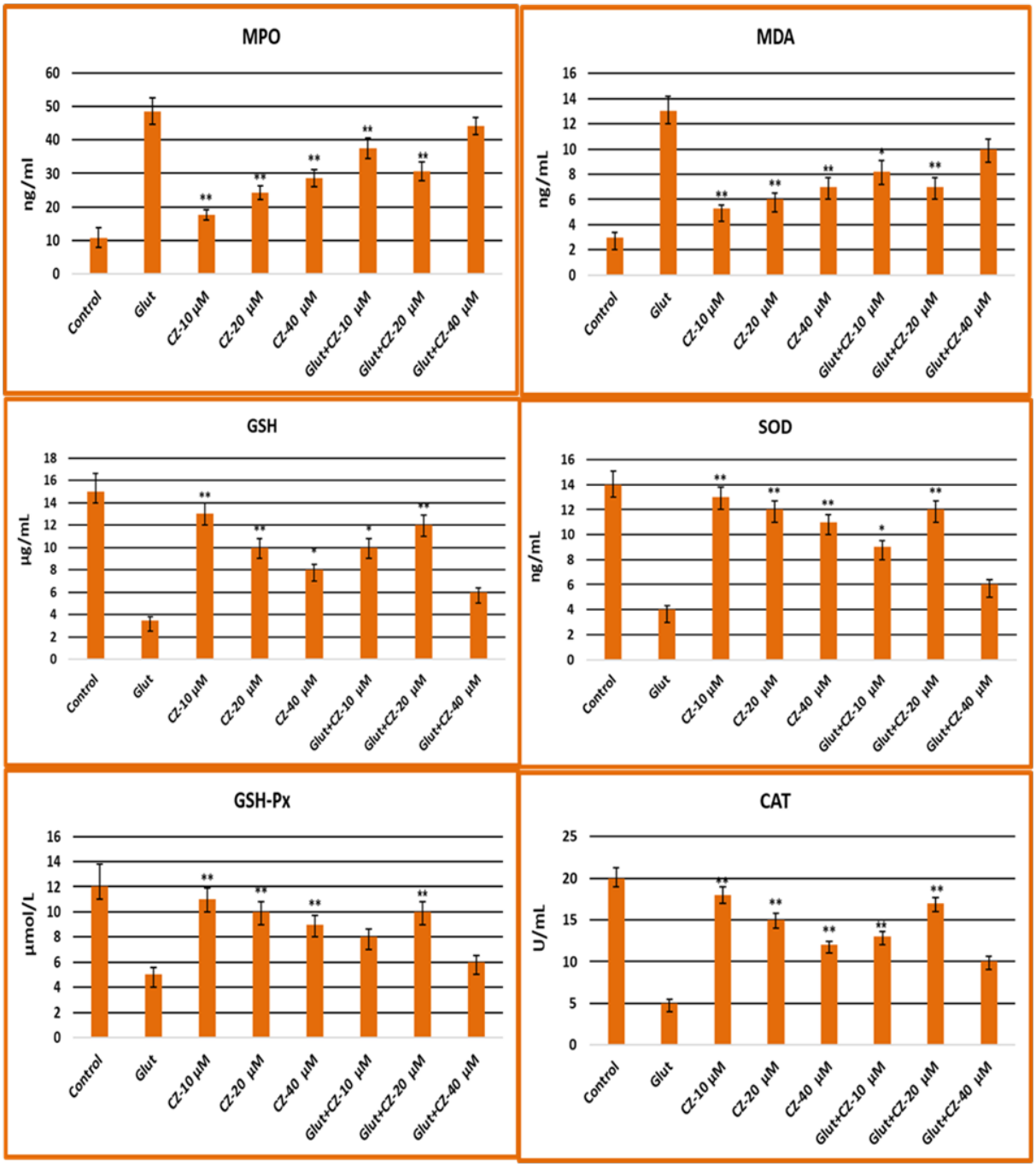

Figure 2: Effects of CZ on the GPx, GSH, MPO, CAT, MDA, and SOD. Data are expressed as the mean ± standard deviation. *$p<.05$ Vs. Glut group, **$p<.001$ Vs. Glut group.

Table 2: Statistical results on the effect of CZ on oxidative stress.

	MPO		MDA		GSH		SOD		GPx		CAT	
Groups	**mean±SD**	***p***	**mean±SD**	***p***	**mean±SD**	***p***	**mean±SD**	***p***	**mean±SD**	***p***	**mean±SD**	***p***
Control	10.79±2,9	0.068	3±0.45	0.102	15.12±1.64	0.234	14±1.12	0.289	12±1.85	0.184	20±1.32	0.312
Glut	48.54±4.3		13±1.25		3.5±0.3		4±0.35		5±0.64		7.23±0.51	
CZ-10 µM	17.58±1.57**	<0.001	5.25±0.32**	<0.001	13±1**	<0.001	13.3±0.87**	<0.001	11±0.95**	<0.001	18.01±1.1**	<0.001
CZ-20 µM	24.17±2**	<0.001	6.15±0.54**	<0.001	10.14±0.8**	<0.001	12.46±0.75**	<0.001	10±0.82**	<0.001	15.2±0.8**	<0.001
CZ-40 µM	28.7±2.5**	<0.001	7±0.74**	<0.001	8.90±0.5*	0.003	11±0.69**	<0.001	9±0.79**	<0.001	12.6±0.43**	<0.001
Glut+CZ-10 µM	37.6±3**	<0.001	8.19±0.9*	0.019	10±0.89*	0.016	9±0.56*	0.035	8±0.69	0.091	13.5±0.66**	<0.001
Glut+CZ-20 µM	30.5±2.8**	<0.001	7.51±0.78**	<0.001	12.27±0.9**	<0.001	12±0.79**	<0.001	10±0.83**	<0.001	17.12±0.7**	<0.001

*$p<.05$ Vs. Glut group, **$p<.001$ Vs. Glut group.

cascades.[21] Glut toxicity plays an important role in the pathogenesis of various neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's and multiple sclerosis.[4] CZ, a benzoxazole derivative; It acts on the central nervous system to relax stiff muscles.[13] However, there is no study in the literature examining the neuroprotective effect of CZ against Glut toxicity. This study is a first in the literature in examining the cytotoxic and antioxidant effects of CZ on primary cortical neuron cells exposed to Glut toxicity. Mitochondrial activity, as assessed by the MTT test, plays a crucial role in cell apoptosis. In our study, CZ treatment reversed the effect of Glut on the survival of cultured neuronal cells in vitro and reduced the inhibition of proliferation, suggesting that CZ could preserve the mitochondrial form.

Glut plays a critical role in neuroinflammation.[22] It may join the neurodegeneration process by way of the release of proinflammatory cytokines, inducible nitric oxide synthase and nitric oxide, reactive oxygen species (ROS), and superoxide levels, which have harmful effects on glutamatergic nerve cells.[23, 24] High levels of MDA, a marker of membrane lipid peroxidation and oxidative stress cause cellular harm through the peroxidation of membrane phospholipids.[25, 26] The rise in MDA activity may be because of the inadequacy of the protective antioxidant molecule GSH.[27] In this study, Glut induction led to an important rise in lipid peroxide amounts. However, the CZ supplement reversed lipid peroxide amounts in Glut-induced groups. The reduction in lipid peroxidation indicates the ability of CZ to scavenge various ROS, including superoxide, peroxyl radical, and hydroxyl radical. In addition, the level of MPO, an inflammation marker, was significantly reduced with CZ treatment.

GSH is the main antioxidant that buffers ROS in the brain. Eliminates H_2O_2 and organic peroxides with GPx. In our work, Glut exposure caused an important loss in the ingredient of antioxidant molecules GPx and GSH because of the overproduction of ROS, which led to oxidative harm to membrane lipids. This oxidative injury is coherent with former reports.[28,29] Moreover, the CZ supplements renovated the amounts and activities of GSH and GPx in Glut-induced neurons, indicating the antioxidant role of CZ. However, SOD and CAT activities decreased significantly with Glut and the activities of these enzymes returned to normal with CZ treatment. These results show that CZ significantly reduces oxidative stress and increases antioxidant activity in neuronal cells.

The fact that only cerebral cortex neurons were examined in our study and that neurons in other parts of the brain were not evaluated constitute the limitations of the study.

CONCLUSION

Based on the data we obtained, CZ showed its neuroprotective effect by attenuating the neuroinflammation caused by Glut. Based on the results, it can be used as a therapeutic agent against Glut-associated neurotoxicity. There is no literature information explaining the relationship between CZ and Glut toxicity, and our study is the first study in this field. In order to better understand the effect of CZ on Glut toxicity, further comprehensive studies, both in vitro and in vivo, are needed.

Ethics Committee Approval: This study was made on the principles of the Declaration of Helsinki. Ethical approval was acquired from Ataturk University Experimental Animals Local Ethics Committee with decision number 236 dated 27.10.2022.

Informed Consent: There is no need to obtain informed consent.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept-YY; Design-YY, BC; Supervision-YY, AH; Resources-YY, BC; Materails-YY, BC, AH; Data Collection and/or Processing-YY, BC, AH; Analysis and/or Interpretation- YY, BC, AH; Literature Search- YY; Writing Manuscript-YY; Critical Review-YY.

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