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Cerebrolysin Alleviating Effect on Glutamate-Mediated Neuroinflammation Via Glutamate Transporters and Oxidative Stress

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Abstract

Glutamate, one of the most important excitatory neurotransmitters, acts as a signal transducer in peripheral tissues and endocrine cells. Excessive glutamate secretion has been shown to cause excitotoxicity and neurodegenerative disease. Cerebrolysin is a mixture of enzymatically treated peptides derived from pig brain including neurotrophic factors, like brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF), and ciliary neurotrophic factor (CNTF). The present study investigated the protective effects of cerebrolysin on glutamate transporters (EAAT 1, EAAT 2) and cytokines (IL-1β and IL-10) activity in glutamate-mediated neurotoxicity. Primary cortex neuron culture was exposed to glutamate and successively treated with various cerebrolysin concentrations for 24 and 48 h. Our data showed that cerebrolysin primarily protects neurons by decreasing glutamate concentration in the synaptic cleft. In addition, Cerebrolysin can decrease oxidative stress and neuron cell damage by increasing antioxidant activity and decreasing inflammation cytokine levels.

Keywords EAAT 1 · EAAT 2 · Glutamate · LDH · IL-1β · IL-10

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Introduction

Excitatory amino acids, such as glutamate (Glut), are important neurotransmitters of the central nervous system (Iovino et al. 2020). Glutamatergic neurotransmission is responsible for many cognitive, motor, sensory, and autonomic activities (Patel and McMullen 2017). Maintaining extracellular glutamate levels within a physiological range is crucial to ensure proper neuronal transmission and viability. Therefore, impaired glutamate homeostasis has important neuropathological consequences (Mahmoud et al. 2019). Exogenous Glut has been reported to cause various neurological or neurodegenerative disorders (NDs), including epilepsy (Barker-Haliski and White 2015), multiple sclerosis (Gautier et al. 2015), Alzheimer's disease (AD) (Olajide et al. 2021), Huntington's disease (HD) (Buren et al. 2020), amyotrophic lateral sclerosis (ALS) (Battaglia and Bruno 2018), and Parkinson's disease (PD) (Iovino et al. 2020), but experimental and clinical data is still controversial (Zanfirescu et al. 2019). Literature data show that neuronal damage, NDs, and many metabolic diseases are mechanistically associated with redox imbalance and oxidative stress (Gubandru et al. 2013; Ungurianu et al. 2019a, b; Ungurianu et al. 2019a, b). Glutamate uptake occurs via five specific glutamate transporters, identified as excitatory amino acid transporters (EAATs) (EAATs 1–5). Two of these, EAAT 1 and EAAT 2 (referred to as GLAST and GLT-1 in the rodent brain), are considered the most important glutamate transporters in the CNS in terms of function and distribution. EAAT 1 is mainly expressed in astrocytes, while EAAT 2, responsible for 90% of brain glutamate reuptake, is highly expressed in astrocytes and neurons (Parkin et al. 2020; Olajide et al. 2021). Cerebrolysin (Cer) is a mixture of low molecular weight peptides and amino acids derived from pig brain tissue. Cer contains a balanced mixture of various neurotrophic factors and active ingredients that induce neuroprotection, neuroplasticity, and regeneration to the neuronal cells (Fujisawa et al. 2000); it is recommended as a potential clinical approach for stroke (Staszewski et al. 2022), traumatic brain injury, Alzheimer's, and Parkinson's disease (Garrido et al. 2015). Cer affects multiple molecular pathways; it interacts with receptors (GluR1 and GABA RA/B) and regulates intracellular signaling pathways (PI3K/Akt, NF-κB, JNK, and p38-MAPK), as well as changes in molecular mediators involved in the trophic activity (NGF, BDNF, and IGF-1), glucose transport (GLUT1), inflammation (TNF-α and IL-1β), and neurotransmission (cholinergic, glutamatergic, and GABAergic) (Lu et al. 2013; Makadia and Siegel 2011; Pongwecharak et al. 2009; Haddadi et al. 2017; Ramesh et al. 2010; Rattan et al. 2012). Cer effects on glutamate transporters and any Cer-transporter-cytokine relations are still unknown.

Protection of neuronal cells against glutamate-induced excitotoxicity may be an effective therapeutic approach for neurodegenerative diseases. In the present study, a glutamate toxicity model was induced in neurons obtained from newborn rats and the effects of Cer on neuroinflammation and biochemical pathways associated with this model were investigated.

Materials and Methods

Chemical and Reagents

Hanks' balanced salt solution (HBSS), trypsin-ethylenediaminetetraacetic acid (EDTA), neurobasal medium (NBM), fetal bovine serum (FBS), B27, antibiotics (penicillin, streptomycin, and amphotericin B), glutamate, cerebrolysin, and dimethyl sulfoxide (DMSO) were obtained from Sigma (USA). Total Antioxidant Status Kit (TAC) and Total Oxidant Status (TOS) were obtained from Rel Assay Diagnostics® Company (Gaziantep, Turkey). Lactate dehydrogenase (LDH) assay was performed using a kit from Cayman Chemicals (USA). 8-OHdG, FITC, and DAPI (Cat no.: D1306) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany).

Ethical Approval

This study was conducted at the Medical Experimental Research Center at Ataturk University (Erzurum, Turkey). The animal use protocol was reviewed and approved by the Ethical Committee of Ataturk University, study protocol 04–2,100,268,999/30.09.2021.

Cell Culture

Primary Neuron Culture

Twelve Sprague Dawley rats, at less than 24 h of birth, were used to obtain cortex neurons; briefly, after the rats were rapidly decapitated, the removed cortexes were transferred to 5 ml of Hanks' balanced salt solution (HBSS), macrofragmented with a scalpel, and then microfragmentation with EDTA (0.25% trypsin–0.02% EDTA). The cells were centrifuged at 1200 rpm for 5 min. Cellular medium for cells (88% Neurobasal Medium (NBM)), with 10% fetal bovine serum (FBS), 2% B27 (Supplement, Thermo Fisher, Germany), 0.1% antibiotic (penicillin–streptomycin), and amphotericin B (Thermo Fisher, Germany) was prepared and cells were seeded in 96-well, 24-well, and 6-well plates. Cells were incubated for 10 days at 5% CO_2 and 37 °C.

Drug Treatment

The neuron cells were treated with glutamate (10^{-5} mM) and glutamate (10^{-5} mM) + Cer (4, 8, 16, and 32 µgr/ml) respectively for 24 and 48 h (5% CO_2, 95% humidity, and 37 °C).

MTT (3-(4,5-Dimethylthiazol-2-yl) Tetrazolium Assay

The neuron cell was seeded in 96-well plates (12 wells for each group). The MTT assay was performed with a commercially available kit (Sigma-Aldrich, USA). Briefly, MTT (10 µL at a concentration of 5 mg/ml) was added to each well and incubated for 4 h (5% CO_2; 37 °C) (Taghizadehghalehjoughi et al. 2019a, b); then, DMSO was added to each well to dissolve formazan crystals. Cell viability (%) was calculated by optical density (OD) read at 570 nm using the Multiskan™ GO Microplate Spectrophotometer reader (Thermo Scientific, California, USA). The OD of the control group was set as 100, and the viability rate of the other groups was calculated according to the formula below.

$$\text{Viability rate}(\%) = (\text{OD of groups}/\text{Control OD}) \times 100$$

Total Antioxidant Capacity (TAC) Assay

The antioxidant capacity was determined by inhibition of the 2–2′-azinobis 3-ethylbenzothiazoline 6-sulfonate ($ABTS^+$) radical cation formation. TAC was measured colorimetrically using the Total Antioxidant Status Kit. Briefly, the initial absorbance of sample-buffer mixture was measured at 660 nm. Then, the ABTS radical cation solution was added, and after 10 min, the second measurement was made at 660 nm. TAC values were calculated as mmol Trolox Equiv./L, according to the formula (YILMAZ et al. 2021):

$$A2 - A1 = \Delta Absorbance\left(\text{standard, sample, or } H_2O\right)$$

$$\text{TAC} = \frac{H_2O\Delta\text{Abs} - \text{Sample } \Delta\text{Abs}}{H_2O\Delta\text{Abs} - \text{Standard } \Delta\text{Abs}}$$

Total Oxidant Status (TOS) Assay

TOS assay is evaluated spectrophotometrically, depending on the number of oxidants in the sample (Taghizadehghalehjoughi et al. 2019a, b). Briefly, the initial absorbance value of sample-buffer mixture was recorded at 530 nm. Then, the Prochromogen solution was added and the second absorbance value was read at 530 nm. TOS values were calculated as mmol H_2O_2 Equiv./L, according to the formula $A2–A1 = \Delta$Absorbance (standard or sample).

$$\text{TOS} = \frac{\text{Sample } \Delta\text{Abs}}{\text{Standard } \Delta\text{Abs}} \times 10$$

Lactate Dehydrogenase (LDH) Assay

LDH was determined with the LDH detection kit, according to the manufacturer's instructions. Cells were seeded in a 96-well plate at a density of 10^3–10^6 cells/well in 200 µL of the medium. Six wells were prepared for each concentration. Triton X-100 (10%) and assay buffer were added and the wells were incubated at room temperature for 1 h. After centrifugation, the cell supernatant was transferred to a new 96-well assay plate. LDH reaction solution was added to each well and the plate was incubated with gentle shaking on an orbital shaker for 30 min at 37 °C. A microplate reader was used to measure the absorbance OD value at 490 nm.

$$\frac{(\text{Experimental value A490}) - (\text{Spontaneous release } A490)}{(\text{Maximum release A490}) - (\text{Spontaneous release } A490)} \times 100$$

*Maximum release: The cells were killed by adding Triton X-100. Spontaneous release: control group with nontoxic materials (cell medium) and experiment value.

Immunofluorescence Staining

After neuron cells fixation in paraformaldehyde for 24 h, the cells were washed again in PBS for 5 min. Endogenous peroxidase was inactivated by keeping it in 3% H_2O_2 for 5 min. After washing for 5–10 min in PBS, 0.1% Triton-X 100 was incubated for 15 min for penetration, and then for 5 min with protein block compatible with all primary and secondary antibodies to prevent nonspecific background staining. At the end of the incubation, primary antibodies (8-OHdG cat no.: sc-66036, diluent ratio: 1/100, USA) were added at 37 °C for 1 h. After washing with PBS 2 times for 5 min, secondary antibody (FITC Cat no.: ab6717, dilution ratio: 1/500, UK) was added and incubated in the dark for 45 min. After the tissues were washed, the DAPI (Cat no.: D1306, dilution ratio: 1/250, UK) solution was added and kept in the dark for 5 min. Sections were examined with a fluorescent microscope (ZEISS Axio, Germany).

Gene Expression

Total RNA was extracted from neuronal cells. Total RNA was used for synthesizing complementary DNA (cDNA) using a High-Capacity cDNA Reverse Transcription Kit. The sequences of gene-specific PCR primers are listed below. The expression of EAAT 1, EAAT 2, IL-1β, and IL-10, cDNA was determined with Rotor-Gene

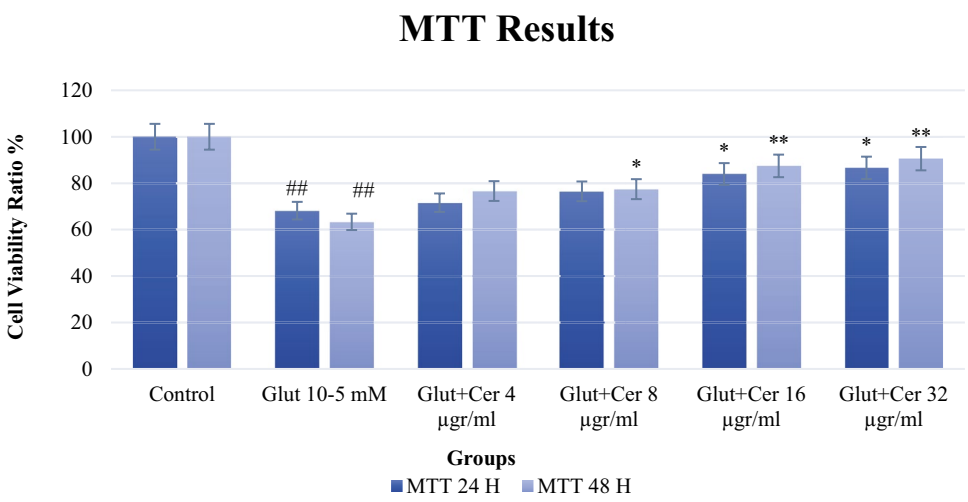

Fig. 1 Cell viability of primary neuron cells at 24 and 48 h. ##Glutamate group compared to control group $P < 0.001$. *, **Glut + Cer (4, 8, 16, and 32 µg/ml) groups compared to glutamate group (*$P < 0.05$, ** $P < 0.001$)

Q (QIAGEN). Taq Man Gene Expression Master Mix Kit was used for PCR amplification and quantification. Results are stated as relative-fold compared with the control group. We normalized gene expressions to beta-actin using the $^{\Delta\Delta}$Ct method (x) and states as fold change to control.

β-Actin:
Forward: 5′-CCAACCGCGAGAAGATGA-3′
Reverse: 5′-CCAGAGGCGTACAGGGATAG-3′
EAAT 1:
Forward: 5′-ACG GTC ACT GCT GTC ATT G-3′
Reverse: 5′-TGT GAC GAG ACT GGA GAT GA-3′
EAAT 2:
Forward: 5′-CCAGTGCTGGAACTTTGCCT-3
Reverse: 5′-TAAAGGGCTGTACCATCCAT-3′
IL-1β:
Forward: 5′-TCTCAGATTCACAACTGTTCGTG-3′
Reverse: 5′-AGAAAATGAGGTCGGTCTCACTA-3′
IL-10:
Forward: 5′-GGCATGCTTGGCTCAGCACTG-3′
Reverse: 5′-GCCCTGCAGTCCAGTAGACG-3′

Statistical Analysis

Statistical comparison between groups was calculated using one-way ANOVA and Tukey HSD method. All calculations were performed using the SPSS 20 software for statistical analysis, and $P < 0.05$ was considered a statistically significant difference in all tests. Results are presented as mean and standard deviation (mean ± SD).

Results

MTT Results

The cytotoxic effect of glutamate 10^{-5} mM (Glut group) and the neuroprotective effect of Cer (4, 8, 16, and 32 µgr/

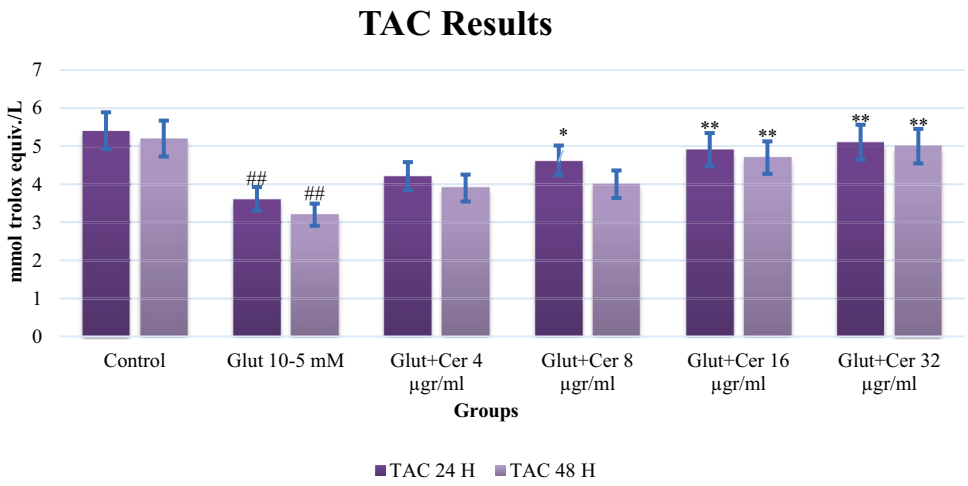

Fig. 2 TAC of primary neuron cells both at 24 and 48 h. ## Glutamate group compared to control group $P < 0.001$. *, **Glut + Cer (4, 8, 16, and 32 µg/ml) groups compared to glutamate group (*$P < 0.05$, **$P < 0.001$)

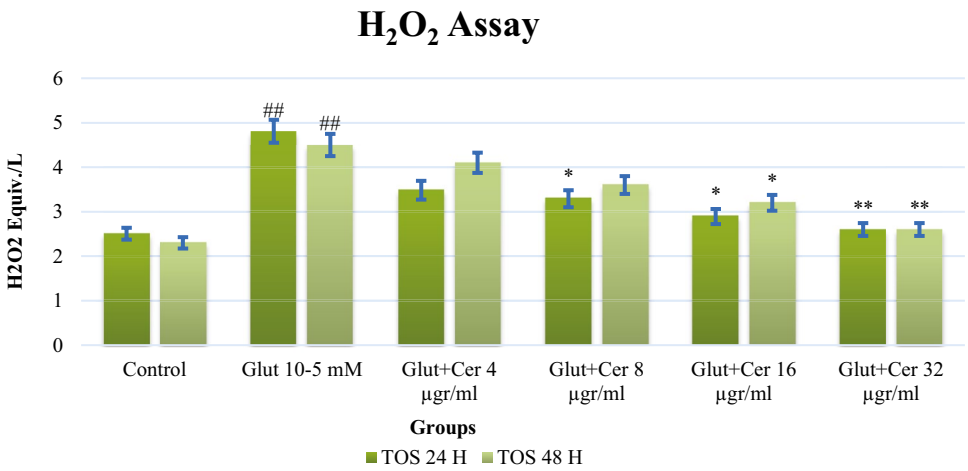

Fig. 3 Total antioxidant capacity of primary neuron cells both 24 and 48 h. The glutamate control compared with the control group (##$P<0.001$: compared to control group). Glut + Cer (4, 8, 16, and 32 µg/ml) is compared with the glutamate group (*$P<0.05$, **$P<0.001$)

ml) on Glut 10^{-5} mM exposed cells (Cer groups) was determined using the MTT method after 24 and 48 h (Fig. 1). A control group was also used for comparison with the glutamate control group. The cell viability ratio of the control group is 100%. The viability ratio of the Glut group is 68.17 and 63.35% for 24 and 48 h, respectively, compared to the control group ($P<0.001$). In the Cer groups, the cell viability ratio increases with increasing Cer concentration compared to the Glut group. The highest viability rate is found at 32 µg/ml Cer (86.61 and 90.56% for 24 and 48 h, respectively).

Redox State in Primary Neurons Treated with Cerebrolysin After Glutamate Toxicity

The total antioxidant capacity (TAC) levels of the control group are constant both at 24 and 48 h, namely 5.4 and 5.2 mmol Trolox Equiv./L, respectively. There is a significant decrease in TAC in the Glut group by 33% and 38% at 24 and 48 h, respectively, which is partly restored at the Cer groups of 4 and 8 µg/ml and almost fully restored at Cer group of 32 µg/ml (6.45% and 1.94% decrease compared to control group at 24 and 48 h, respectively) (Fig. 2).

Total oxidative status (TOS) levels were evaluated in mmol H_2O_2 Equiv./L (Fig. 3). The oxidant level of the control group was found to be 2.3 and 2.5 mmol H_2O_2 Equiv./L at 24 and 48 h, respectively. After the glutamate application, this value was found to be 4.8 at 24 h and 4.5 mmol H_2O_2 Equiv./L at the end of 48 h ($P<0.001$ in comparison to control group). The lowest oxidation rate among treatments was seen in the Glut 10^{-5} mM + Cer 32 µg/ml group (2.6 mmol H_2O_2 Equiv./L in both hours) ($P<0.001$). The application of Cer at different doses after exposure to glutamate showed that Cer protects neuron cells against glutamate toxicity and the TOS value approached the control group (Fig. 3).

LDH Dynamics for Primary Neurons Treated with Cerebrolysin After Glutamate Toxicity

The activity of LDH was rated according to the standard solution (pure-%100) of the commercial kit (Fig. 4). The LDH level of the control group was 9% at the end of 24 h

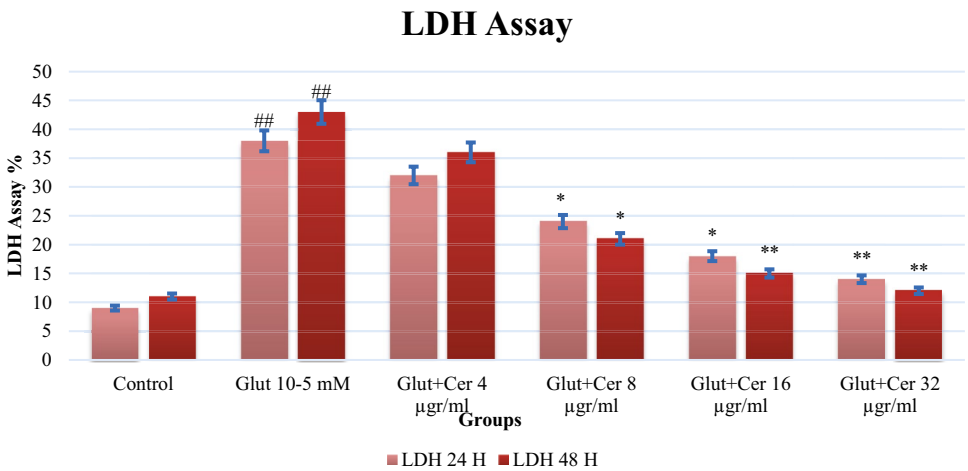

Fig. 4 LDH activity of primary neuron cells for both 24 and 48 h. The glutamate control compared with the Control group (##$P<0.001$: compared to control group). Glut + Cer (4, 8, 16, and 32 µg/ml) is compared with the glutamate group (*$P<0.05$, **$P<0.001$)

and 11% at the end of 48 h. LDH shows an increase in the Glut group, 38% for the first 24 h and 43% at the end of 48 h. The LDH level decreased sharply in the treatment groups (Glut 10^{-5} mM + Cer 4 µg/ml (32% and 36%), 8 µg/ml (24% and 21%), 16 µg/ml (18 and 15%), respectively) and the most effective result was seen in the Cer 32 µg/ml group 12% ($P < 0.001$).

Immunofluorescence Analysis

A significant decrease in cell viability and signs of inflammation were observed in the Glut group. The Cer (4 to 32 µg/ml) increased the viability ratio and the inflammation decreased (Fig. 5). Data of immunofluorescence staining findings and statistical analysis results are given in Table 1.

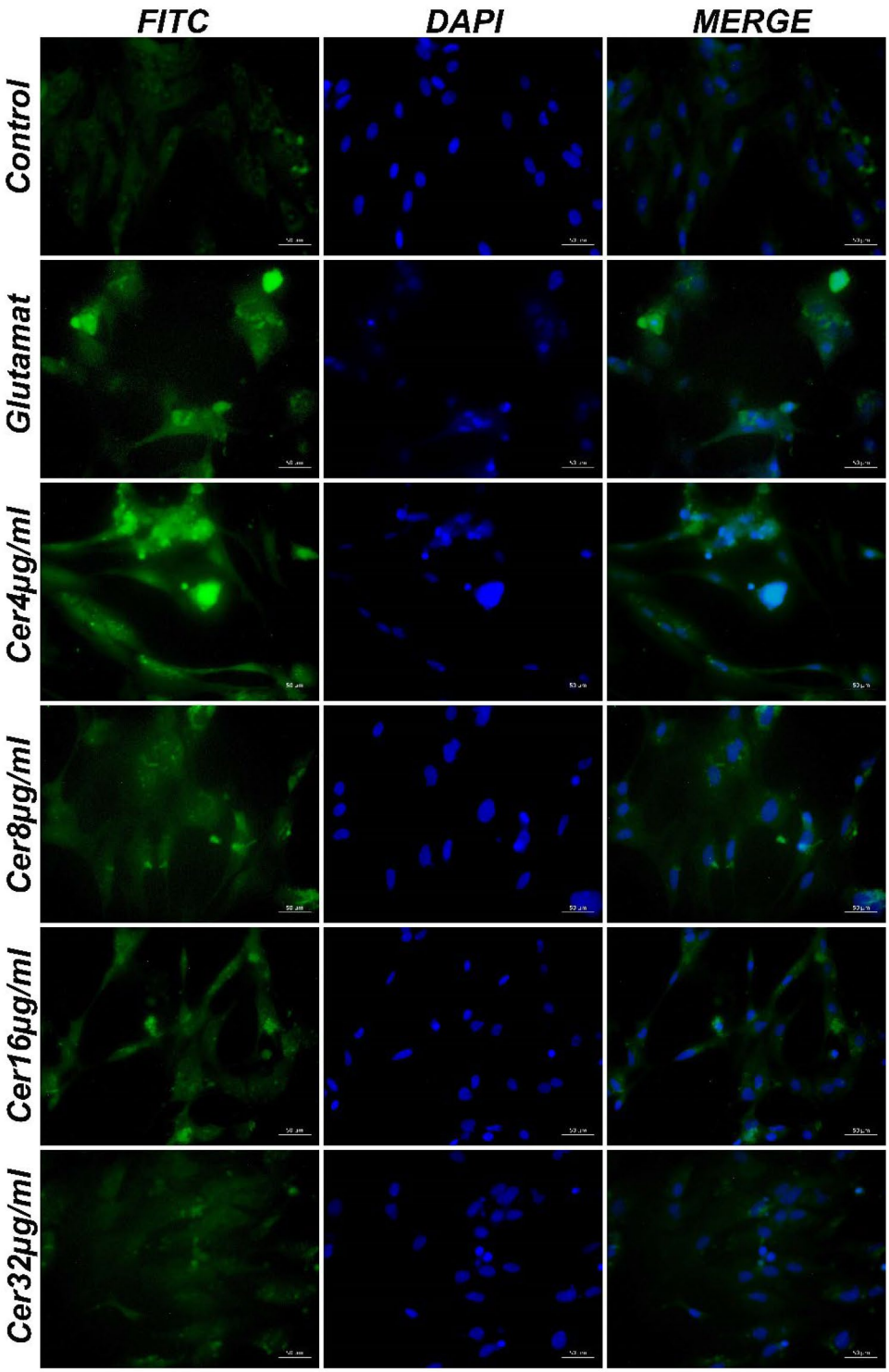

Fig. 5 8-OHdG, DAPI, and MERGE images of experimental groups. Immunofluorescence images of control, Glut 10^{-5} mM, and Glut 10^{-5} mM + Cer 4, 8, 16, and 32 µg/ml 48 h after treatment

Table 1 Scoring and analysis of 8-OHdG immunofluorescent staining. The groups were control, Glut 10^{-5} mM, and Glut 10^{-5} mM + Cer 4, 8, and 32 µg/ml 48 h after treatment. a, b, c, d: different letters in the column represent statistically ($P < 0.05$) significant difference

Groups	8-OHdG
Control	24.75 ± 1.86^{a}
Glut 10^{-5} mM	104.55 ± 2.18^{b}
Glut 10^{-5} mM + Cer 4 µg/ml	100.33 ± 2.49^{b}
Glut 10^{-5} mM + Cer 8 µg/ml	74.8 ± 2^{c}
Glut 10^{-5} mM + Cer 16 µg/ml	57.68 ± 1.24^{d}
Glut 10^{-5} mM + Cer 32 µg/ml	27.57 ± 1.92^{a}

Real-Time PCR Results

Glut + Cer Changes IL-1β Gene Expression in Neuron

Neuron's cell IL-1β gene expression level was given in Fig. 6. It is observed that IL-1β production in neuronal cells increases as a result of glutamate administration. IL-1β overexpression is a marker of severe inflammation in neuronal cells. The data shows statistical difference when the glutamate control group is compared with the control ($P < 0.001$). This data shows the central role of IL-1β in mediating neuroinflammation (correlation with MTT results). In response to induced glutamate toxicity, there was a decrease in IL-1β levels in the cerebrolysin-administered groups. Compared to the Glut 10^{-5} mM group, the most effective result (decrease in IL-1β expression) was observed in the Glut 10^{-5} mM + Cer 16 and 32 µg/ml groups ($P < 0.001$).

Glut + Cer Changes IL-10 Expression in Neuron

Interleukin-10 is involved in both immunoproliferative and inflammatory suppression by inhibiting the release of proinflammatory cytokines. Therefore, an imbalance between proinflammatory and anti-inflammatory cytokines may be an important phenomenon in AD. This hypothesis is supported by studies that describe a 7- to tenfold increase in IL-1β production relative to IL-10 levels in AD patients. Similarly, in our study, in contrast to IL-1β, there was a serious decrease in IL-10 levels in cells exposed to Glut 10^{-5} mM (Fig. 7) ($P < 0.01$). Contrary to IL-1β data, the Glut 10^{-5} mM + Cer 32 µg/ml group upregulated the IL-10 level that was statically significant ($P < 0.001$).

Glut + Cer Did Not Affect Expression of Excitatory Amino Acid Transporters in Neuron

Excitatory amino acid transporters 1 and 2 (EAAT 1 and EAAT 2) are the main mediators for glutamate clearance in humans (Fig. 8). The normal physiological function of EAATs 1 and 2 is the clearance of the glutamate from synapses. Impaired glutamate uptake with dysfunction or reduced expression of EAATs 1 and 2 has been implicated in the pathogenesis of several neurodegenerative diseases such as AD. In our study, there was a decrease in EAAT 1 ($P < 0.05$) and no change in EAAT 2 ($P > 0.05$) gene expression level in the Glut 10^{-5} mM group compared to the control group. However, EAAT 1 expression level was increased by increased Cer doses after Glut 10^{-5} mM application. EAAT 1 gene expression was the highest in Glut 10^{-5} mM + Cer 32 µg/ml ($P < 0.05$). Although the EAAT 2 gene level, there was no detected change in treatment groups ($P > 0.05$). The slight increase in the EAAT 2 level after Glut 10^{-5} mM + Cer 32 µg/ml administration was not significant.

Discussion

Glutamate is an important excitatory neurotransmitter and is rapidly removed from the synaptic cleft under physiological settings. Glutamate-induced neurotoxicity is

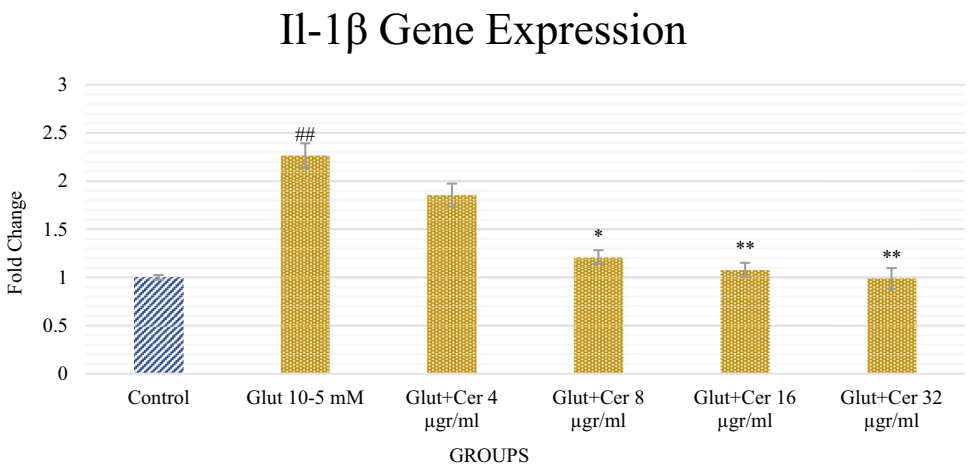

Fig. 6 IL-1β gene expression was shown. The groups were control, Glut 10^{-5} mM, and Glut $10.^{-5}$ mM + Cer 4, 8, and 32 µg/ml 48 h after treatment (*$P < 0.05$, **$P < 0.001$: compared to the glutamate group, ## $P < 0.001$: compared to the control group)

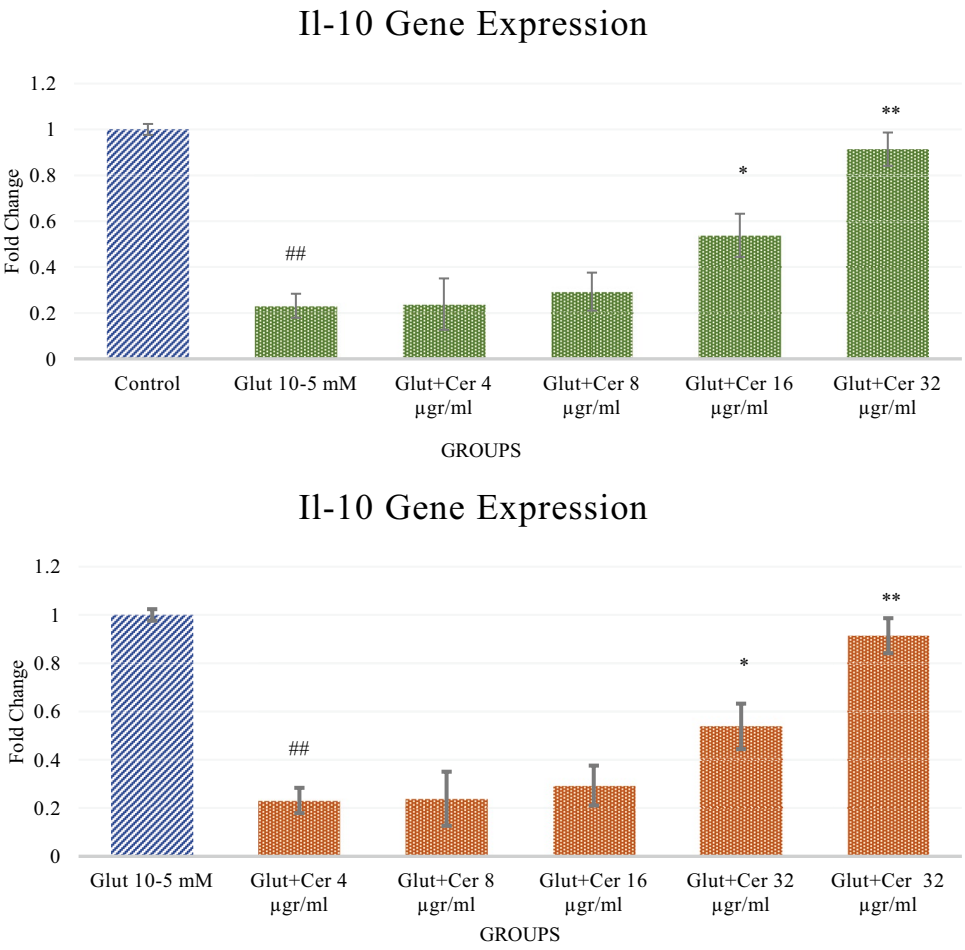

Fig. 7 IL-10 gene expression was shown. The groups were control, Glut 10^{-5} mM, and Glut $10.^{-5}$ mM + Cer 4, 8, and 32 µg/ml 48 h after treatment (*$P < 0.05$, **$P < 0.001$: compared to glutamate group, ## $P < 0.001$: compared to control group)

involved in the pathophysiology of many diseases such as epilepsy, multiple sclerosis, Alzheimer's disease (AD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and Parkinson's disease (PD) (Lewerenz and Maher 2015). However, due to the inability of the transporters to remove the accumulation of glutamate, it has been shown in many literature references that the accumulation of glutamate leads to overstimulation of

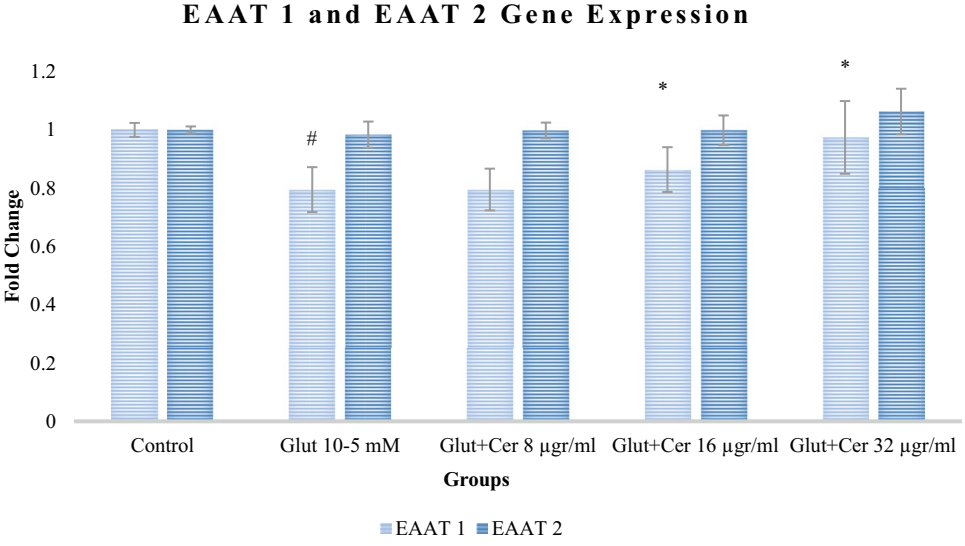

Fig. 8 EAAT 1 and EAAT 2 gene expression levels were shown. The groups were control, Glut 10^{-5} mM, and Glut $10.^{-5}$ mM + Cer 4, 8, and 32 µg/ml 48 h after treatment (*$P < 0.05$ compared to glutamate group, ##$P < 0.05$: compared to control group)

the receptors and cell death (Iovino et al. 2020; Deletage et al. 2021; Song et al. 2020; Taghizadehghalehjoughi and Naldan 2021). The overstimulation of glutamate receptors causes DNA damage and leads to the release of free radicals. Cerebrolysin is a peptide with a pharmacological effect similar to endogenous neurotrophic factors. In our study, we investigated the effect of Cer peptides on glutamate toxicity.

MTT results show glutamate toxicity causes neuronal cell death (68%). Although the mechanisms of glutamate-induced cell death described in various studies are different. The cell death occurs due to damage to some cell compartments, including mitochondria (Atlante et al. 2001). Mitochondria and cell compartment damage cause reactive oxygen species (ROS) to produce and also irreversible damage including DNA fragmentation (Yeni et al. 2022). Cer was used for the elimination of glutamate excitotoxicity.

The oxidative damage was reduced in the Cer-administered groups and the antioxidant activity increased especially at 16 and 32 µg/ml. We think, Cer increase cellular viability by directly effect on the ROS mechanism. Cer, a combination of neurotrophic factors, not only reduces oxidative stress but also leads to increase antioxidant enzyme activities (Vaghef et al. 2019; Alzoubi et al. 2020). The Cer reduces glutamate-induced memory dysfunction, at least in part, by inhibiting oxidative damage and cell death in neurons.

Astrocytic glutamate transporters, EAATs 1 and 2, play an important role in eliminating released glutamate from the synapse cleft (re-uptake). Elongation glutamate re-uptake time (glutamate transporter insufficiency and dysfunction) induced excitotoxic injury and cellular death (Karki et al. 2018). Dysregulation of EAAT 1 contributes to the pathogenesis of multiple neurological disorders such as AD, ataxia, traumatic brain injuries, and glaucoma (Maragakis and Rothstein 2004; Hanani 2005). In our study, the cerebrolysin effects on EAATs 1 and 2 in glutamate-induced toxicity were determined. We found different effects on EAAT 1 and EAAT 2 transporters. A significantly increase was seen in the expression and function of EAAT 1 but EAAT 2 did not change meaningly. As a result, while EAAT 1 was expressed low in the presence of glutamate, its expression level increased after Cer application. SA significant decrease in the IL-1β expression level was shown at 16 and 32 µg/ml Cer. Similarly, using an ischemic brain model, Cer injection induced a significant decrease in the IL-1β level. Cer protects neurons by increasing antioxidant capacity and decreasing LDH levels (Guan et al. 2019). In parallel, Cer increases the level of IL-10 and supports the anti-inflammatory process. EIL-10 has an important protective role against brain damage and is a marker for cell recovery (Garcia et al. 2017). In addition, in the immunofluorescence experiment conducted to evaluated the expression of 8-OHdG. The 8-OHdG expression was increased in the glutamate control group due to DNA damage induction. 8-OHdG was used in nuclear and mitochondrial DNA and is widely used as a biomarker for oxidative stress and oxidative related to DNA damage (Valavanidis et al. 2009). The 8-OHdG level by adding Cer shows decrease especially in high doses due to decrease in the TOS and LDH levels. According to our data, the 8-OHdG level shows correlation with IL-1β, TOS, and MTT results. In addition, the transporter activity leads to decrease in 8-OHdG expression levels.

Conclusion

In our study, the effects of various Cer concentrations, when glutamate toxicity has already been established after relevant pre-exposure, were investigated. It seems that Cer exerts a protective effect at doses of 16 and 32 µg/ml, by preventing neuroinflammation and attenuating oxidative damage, as shown by the significant increase in IL-10 and TAC levels observed. Cer also enhances glutamate clearance, as depicted by the increase on the EAAT 1 gene expression level in the Cer groups. In conclusion, Cer could be recommended for the prevention of glutamate-related neurodegenerative diseases such as Alzheimer's.

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Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics Approval This study was conducted at the Medical Experimental Research Center at Ataturk University (Erzurum, Turkey). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Ethical Committee of Ataturk University, study protocol 04–2100268999/30.09.2021.

Consent for Publication No conflict of interest exists in the submission of this manuscript, and this manuscript is approved by all authors for publication.

Competing Interests The authors declare no competing interests.

References

Alzoubi KH, Al-Jamal FF, Mahasneh AF (2020) Cerebrolysin prevents sleep deprivation induced memory impairment and oxidative stress. Physiol Behav 217

Atlante A, Calissano P, Bobba A, Giannattasio S, Marra E, Passarella S (2001) Glutamate neurotoxicity, oxidative stress and mitochondria. FEBS Lett 497:1–5

Barker-Haliski M, White HS (2015) Glutamatergic mechanisms associated with seizures and epilepsy. Cold Spring Harb Perspect Med 5

Battaglia G, Bruno V (2018) Metabotropic glutamate receptor involvement in the pathophysiology of amyotrophic lateral sclerosis: new potential drug targets for therapeutic applications. Curr Opin Pharmacol 38:65–71

Buren C, Tue GQ, Raymond LA (2020) Impaired replenishment of cortico-striatal synaptic glutamate in Huntington's disease mouse model. J Huntington's Dis 9:149–161

Deletage N, Le Douce J, Callizot N, Godfrin Y, Lemarchant S (2021) SCO-spondin-derived peptide protects neurons from glutamate-induced excitotoxicity. Neuroscience 463:317–336

Fujisawa H, Reis RM, Nakamura M, Colella S, Yonekawa Y, Kleihues P, Ohgaki H (2000) Loss of heterozygosity on chromosome 10 is more extensive in primary (de novo) than in secondary glioblastomas. Lab Invest 80:65–72

Garcia JM, Stillings SA, Leclerc JL, Phillips H, Edwards NJ, Robicsek SA, Hoh BL, Blackburn S, Dore S (2017) Role of interleukin-10 in acute brain injuries. Front Neurol 8:244

Garrido P, Osorio FG, Moran J, Cabello E, Alonso A, Freije JM, Gonzalez C (2015) Loss of GLUT4 induces metabolic reprogramming and impairs viability of breast cancer cells. J Cell Physiol 230:191–198

Gautier HOB, Evans KA, Volbracht K, James R, Sitnikov S, Lundgaard I, James F, Lao-Peregrin C, Reynolds R, Franklin RJM, Karadottir RT (2015) Neuronal activity regulates remyelination via glutamate signalling to oligodendrocyte progenitors. Nat Commun 6

Guan X, Wang Y, Kai G, Zhao S, Huang T, Li Y, Xu Y, Zhang L, Pang T (2019) Cerebrolysin ameliorates focal cerebral ischemia injury through neuroinflammatory inhibition via CREB/PGC-1alpha pathway. Front Pharmacol 10:1245

Gubandru M, Margina D, Tsitsimpikou C, Goutzourelas N, Tsarouhas K, Ilie M, Tsatsakis AM, Kouretas D (2013) Alzheimer's disease treated patients showed different patterns for oxidative stress and inflammation markers. Food Chem Toxicol 61:209–214

Haddadi N, Lin Y, Simpson AM, Nassif NT, McGowan EM (2017) Dicing and splicing sphingosine kinase and relevance to cancer. Int J Mol Sci 18

Hanani M (2005) Satellite glial cells in sensory ganglia: from form to function. Brain Res Rev 48:457–476

Iovino L, Tremblay ME, Civiero L (2020) Glutamate-induced excitotoxicity in Parkinson's disease: the role of glial cells. J Pharmacol Sci 144:151–164

Karki P, Hong P, Johnson J Jr, Pajarillo E, Son DS, Aschner M, Lee EY (2018) Arundic acid increases expression and function of astrocytic glutamate transporter EAAT1 via the ERK. Akt NF-kappaB Pathways Mol Neurobiol 55:5031–5046

Lewerenz J, Maher P (2015) Chronic glutamate toxicity in neurodegenerative diseases-what is the evidence? Front Neurosci 9:469

Lu WR, Defilippi J, Braun A (2013) Unleash metformin: reconsideration of the contraindication in patients with renal impairment. Ann Pharmacother 47:1488–1497

Mahmoud S, Gharagozloo M, Simard C, Gris D (2019) Astrocytes maintain glutamate homeostasis in the CNS by controlling the balance between glutamate uptake and release. Cells 8

Makadia HK, Siegel SJ (2011) Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier. Polymers (basel) 3:1377–1397

Maragakis NJ, Rothstein JD (2004) Glutamate transporters: animal models to neurologic disease. Neurobiol Dis 15:461–473

Olajide OJ, Gbadamosi IT, Yawson EO, Arogundade T, Lewu FS, Ogunrinola KY, Adigun OO, Bamisi O, Lambe E, Arietarhire LO, Oluyomi OO, Idowu OK, Kareem R, Asogwa NT, Adeniyi PA (2021) Hippocampal degeneration and behavioral impairment during Alzheimer-like pathogenesis involves glutamate excitotoxicity. J Mol Neurosci 71:1205–1220

Parkin GM, Gibbons A, Udawela M, Dean B (2020) Excitatory amino acid transporter (EAAT)1 and EAAT2 mRNA levels are altered in the prefrontal cortex of subjects with schizophrenia. J Psychiatr Res 123:151–158

Patel RAG, McMullen PW (2017) Neuroprotection in the treatment of acute ischemic stroke. Prog Cardiovasc Dis 59:542–548

Pongwecharak J, Tengmeesri N, Malanusorn N, Panthong M, Pawangkapin N (2009) Prescribing metformin in type 2 diabetes with a contraindication: prevalence and outcome. Pharm World Sci 31:481–486

Ramesh M, Ahlawat P, Srinivas NR (2010) Irinotecan and its active metabolite, SN-38: review of bioanalytical methods and recent update from clinical pharmacology perspectives. Biomed Chromatogr 24:104–123

Rattan R, Ali Fehmi R, Munkarah A (2012) Metformin: an emerging new therapeutic option for targeting cancer stem cells and metastasis. J Oncol 2012:928127

Song XR, Gong ZX, Liu KL, Kou JP, Liu BL, Liu K (2020) Baicalin combats glutamate excitotoxicity via protecting glutamine synthetase from ROS-induced 20S proteasomal degradation. Redox Biology 34

Staszewski J, Stępień A, Piusińska-Macoch R, Dębiec A, Gniadek-Olejniczak K, Frankowska E, Maliborski A, Chadaide Z, Balo D, Król B (2022) Efficacy of cerebrolysin treatment as an add-on therapy to mechanical thrombectomy in patients with acute ischemic stroke due to large vessel occlusion: study protocol for a prospective, open label, single-center study with 12 months of follow-up. Front Neurol 13

Taghizadehghalehjoughi A, Naldan ME (2021) Is ketamine suitable for use in glutamate toxicity conditions?: an in vitro study. J Invest Surg 34:121–128

Taghizadehghalehjoughi A, Sezen S, Hacimuftuoglu A, Gulluce M (2019a) Vincristine combination with Ca(+2) channel blocker increase antitumor effects. Mol Biol Rep 46:2523–2528

Taghizadehghalehjoughi A, Hacimuftuoglu A, Yilmaz A (2019b) Na+ channel blocker enhances metformin effects on neuroblastoma cell line. Med Sci 8:636–640

Ungurianu A, Şeremet O, Grădinaru D, Ionescu-Tîrgovişte C, Margină D, Miulescu RD (2019a) Spectrophotometric versus spectrofluorometric assessment in the study of the relationships between lipid peroxidation and metabolic dysregulation. Chem Biol Drug Des 93:1026–1035

Ungurianu A, Şeremet O, Gagniuc E, Olaru OT, Guţu C, Grădinaru D, Ionescu-Tîrgovişte C, Margină D, Dănciulescu-Miulescu R (2019b) Preclinical and clinical results regarding the effects of a plant-based antidiabetic formulation versus well established antidiabetic molecules. Pharmacol Res 150:104522

Vaghef L, Farajdokht F, Erfani M, Majdi A, Sadigh-Eteghad S, Karimi P, Shotorbani SS, Vafaee MS, Mahmoudi J (2019) Cerebrolysin attenuates ethanol-induced spatial memory impairments through inhibition of hippocampal oxidative stress and apoptotic cell death in rats. Alcohol 79:127–135

Valavanidis A, Vlachogianni T, Fiotakis C (2009) 8-Hydroxy-2'-deoxyguanosine (8-OHdG): a critical biomarker of oxidative stress and carcinogenesis. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 27:120–139

Yeni Y, Cakir Z, Hacimuftuoglu A, Taghizadehghalehjoughi A, Okkay U, Genc S, Yildirim S, Saglam YS, Calina D, Tsatsakis

A, Docea AO (2022) A selective histamine H4 receptor antagonist, JNJ7777120, role on glutamate transporter activity in chronic depression. J Pers Med 12

Yilmaz A, Taghïzadehghalehjoughï A, Hacïmuftuoglu A, Türkmen A (2021) Investigation of Aloe vera barbadensis Miller leaf extract effects on glutamate and glyphosate induced toxicity: in vitro study. J Anatol Environ Animal Sci 6:376–381

Zanfirescu A, Ungurianu A, Tsatsakis AM, Nitulescu GM, Kouretas D, Veskoukis A, Tsoukalas D, Engin AB, Aschner M, Margina D (2019) A review of the alleged health hazards of monosodium glutamate. Compr Rev Food Sci Food Saf 18:1111–1134