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Research Paper

Investigation of Antioxidant Efficacy of *Glycyrrhiza glabra* L. Extract in Glutamate Toxicity-Induced Primary Neuron Culture

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Abstract

Glutamate toxicity and reactive oxygen accumulation are life-threatening factors that play a role in the pathogenesis of neurodegenerative diseases. Today, many studies are carried out to elucidate these pathological pathways and to reveal possible treatment possibilities. Herbal medicines have been used continuously by people since ancient times due to their low side effects and easy accessibility. Studies have shown that extracts obtained from *Glycyrrhiza glabra* L. plant have anti-inflammatory, antioxidant, antidiabetic and neuroprotective effects. In this study, it was aimed to investigate the neuroprotective effect of *Glycyrrhiza glabra* L. water extract against glutamate toxicity induced in primary neuron culture. The experiment consists of negative control, positive control and *Glycyrrhiza glabra* L. treatment groups at doses of 100 µg/ml, 200 µg/ml, 400 µg/ml. In this study, glutamate toxicity was induced by exposing all experimental groups to glutamate at a concentration of 10^{-5} M for 5 minutes, except for the negative control group in which primary neuron culture was prepared. After glutamate toxicity, cell viability (MTT), cytotoxicity level (LDH), oxidant (MDA, TOS) and antioxidant (SOD, TAC) status of the experimental groups were investigated using biochemical methods. Obtained results were analyzed statistically. As a result of the analysis, glutamate-induced cell death was decreased in the experimental groups to which *Glycyrrhiza glabra* L. extract was applied. Compared to the positive control group, the level of oxidative stress para *Glycyrrhiza glabra* L. extract. However, it was observed that the group treated with 200 µg/ml *Glycyrrhiza glabra* L. extract showed the best results against glutamate toxicity. In this study, it was determined that *Glycyrrhiza glabra* L. extract reduced glutamate toxicity with antioxidant effect and provided neuroprotective effect.

Keywords: Glutamate toxicity, *Glycyrrhiza glabra* L., Liquorice, Oxidative stress. Primer Neuron culture,

1. Introduction

Glycyrrhiza glabra L. (GG, Licorice) is a perennial herb in the legume family. Extracts obtained from different parts of the plant have been consumed by people for centuries both as food and used for the treatment of various diseases such as stomach, respiratory tract and skin diseases. GG root extracts are still used in the food and pharmaceutical industries today [1]. In the studies, it was reported that the extracts obtained from the GG root contain phytosterols, coumarins, vitamins, triterpenes, saponins and various flavonoids. In the researches, antimicrobial, anticancer, antioxidant, anti-inflammatory, hepatoprotective and other pharmacological effects of GG extracts were associated with its components. As a matter of fact, in studies glabridin was used which one of the major components of GG root extract, it was determined that glabridin was responsible for a significant part of the bioactivities exhibited by these GG root extracts, depending on the application method and dose [2]. When toxicological studies of GG root extract are encountered, it has been reported that its toxicity is dose-related and shows weak mutagenicity, genotoxicity, and carcinogenicity at high doses [3]. Glutamate is the main excitatory neurotransmitter. Glutamatergic synapses constitute 40% of the neurons spread to the central nervous system. Increased extracellular glutamate is known to cause nerve cell death in strokes or traumas [4]. Increased extracellular glutamate cause CNS nerve cell death. Acute CNS injuries such as ischemia and traumatic brain injury have traditionally been the focus of excitoxicity research. However, glutamate excitotoxicity is also linked to chronic neurodegenerative disorders,

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including amyotrophic lateral sclerosis (ALS), multiple sclerosis and Parkinson's disease (PD). Oxidative stress is one of the main factors that cause damage to neurons and cause cell death. Excessive glutamate release can also cause apoptosis by causing oxidative stress. This is due to the inability of the brain to show sufficient antioxidant activity and excessive accumulation of radical oxygen derivatives. Glutamate is responsible for the majority of oxidative stress in the CNS [5, 6]. Therefore, many cell models exploit glutamate toxicity to induce oxidative neuronal death [7]. As a result of excitotoxicity caused by irregular and excessive glutamate release, reactive oxygens are synthesized excessively and energy deficiency occurs due to mitochondrial dysfunction, and this can bring about apoptosis. Glutamate toxicity causes oxidative stress in two ways. First, it activates chemicals leading to glutamate reactive production that binds to N-methyl-D-aspartate, the ionotropic receptor of glutamate [5, 8]. The second way is that glutamate interacts with the cystine transporter, leading to a decrease in glutathione, which acts as an intracellular antioxidant, leading to reactive oxygen accumulation [9, 10]. In this study, the effect of GG extract on cell viability, oxidant and antioxidant levels against in-vitro glutamate toxicity modeled in primary neuron culture was investigated.

2. Material and Method

2.1. Experiment design and applications

This study was approved by Atatürk University Animal Experiments Local Ethics Committee, Turkey (No: E-42190979-000-22000023282). This study consisted of 5 groups as represented at Table 1. Licorice extract was purchased from SepeNatural Izmir Turkey.

NC	Negative Control, Healthy Neuron Culture
PC	Positive Control, Glutamate Toxicity Group
GG 100 µg/ml	Glutamate Toxicity + 100 µg/ml GG extract
GG 200 µg/ml	Glutamate Toxicity + 200 µg/ml GG extract
GG 400 µg/ml	Glutamate Toxicity + 400 µg/ml GG extract

Table 1. Experiment Groups

2.1.1. Primary Neuron Culture

After 10 newborn rat puppies are taken, they are washed with povidone iodine and taken into a sterile petri dish and decapitated quickly. After the head is covered with gauze, the skin and skull are opened with fine scissors, the cortex is taken with the help of a brain knife and transferred to a tube containing 2 cc DMEM (Gibco, USA) solution. After all the cortices are collected, the brains that have sunk to the bottom are taken into a petri dish and micro-smashed with light touches for 20 minutes with the help of a double scalpel. ¼ of trypsin-EDTA (0.25 %; Sigma-Aldrich Co. Ltd., Irvine, UK) is added to the fragmented cortex, which is taken back into the DMEM solution and incubate for 30 minutes. The cells removed from the incubator are centrifuged 3 times at 1200 rpm for 5 minutes. At the end of each centrifuge, the supernatant is discarded and replaced with a new medium. The obtained pure neuron cells are placed in the prepared neuron solution with 1/10 FBS, 1/50 B27, 1/1000 antibiotic (pencillin-streptomycin-amphotricine B) and 1×10^5 cells per well are seeded in 96 polylysine coated polystyrene dishes. Cells are checked and medium changes are made regularly every three days. The plates are left in an incubator with 5% CO_2 at 37°C so that the cells can reconnect [11].

2.1.2. Establishment of Glutamate Toxicity

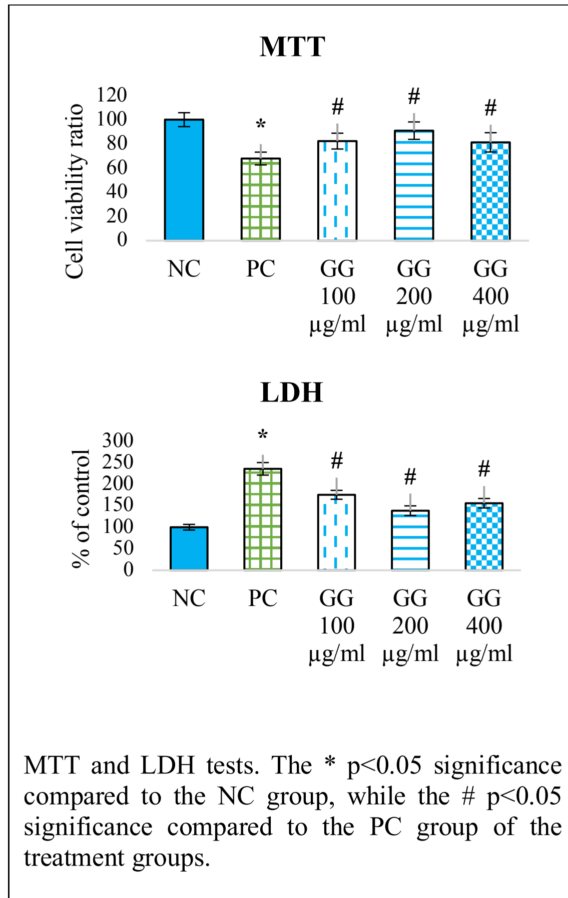
To induce excitotoxic damage, primary cortical neurons are exposed to glutamate at a concentration of 10^{-5} M for 5 minutes. It is then incubated for 24 hours applying different concentrations to evaluate the effect of GG on glutamate excitotoxicity. After the incubation period, cell viability (cytotoxicity status) is evaluated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and lactate dehydrogenase analysis methods [12].

2.2. MTT (3-(4,5-dimetiltriazol-2-il)-2,5-difeniltetrazolyum bromid) Assay

The principle of this method is based on the mitochondrial dehydrogenase enzyme found in the mitochondria of living cells, which oxidizes lactate to pyruvate and converts the yellow MTT reactant of NADH+ to purple formazan. The resulting purple formazan crystals are dissolved and the absorbance is read. The absorbance value is directly proportional to the vitality. Therefore, as the number of viable cells increases, the absorbance becomes higher, and as the viability decreases, the absorbance decreases.

The toxic effects of the polymer and antigens to be used in the experiments are examined in the neuron culture cell. After the neuron cells in the prepared culture are seeded in flat-bottomed 96-well plates and the surface coating of the cells is completed, different concentrations of Control and Treatment drugs are added to the medium. While the cells are in the incubation period, they are checked every 24 hours, and if the surface coating is complete, 10 µl of the prepared MTT solution at a concentration of 10 mg/ml is added to all wells and incubated for 4 hours at 37°C. After the formation of purple formazan crystals is examined and confirmed with an invert microscope, the medium is discharged without damaging the crystals to stop the reaction, and 100 µl of DMSO is added to each well and incubated at room temperature in the dark to check that the formazan crystals are dissolved. Finally,

absorbance is performed with the aid of an ELISA reader tuned to a wavelength of 570 nm [13, 14].



MTT and LDH tests. The * p<0.05 significance compared to the NC group, while the # p<0.05 significance compared to the PC group of the treatment groups.

Figure 1. Viability Level of Primary Neuron Culture

2.3. LDH (Lactat Dehidrogrnase) Assay

To assess cytotoxicity, the lactate dehydrogenase (LDH) level is measured colorimetrically using the manufacturer's kit procedure (LDH assay kit, Elabscience, USA). Optical density was evaluated at 450 nm (BioTek Instruments, USA) [15].

2.4. Oxidative stress markers

To measure oxidative stress levels in primary neuron culture, such as Total Antioxidant Status (TAS) [15], Total Oxidant Status (TOS) (Rel Assay Diagnostics, Gaziantep, Turkey)[14], Malondialdehyde (MDA)[16] and Superoxide dismutase (SOD) (Elabscience, United States)[15] activities were measured using the ELISA kit according to the manufacturer's manual. All absorbance measurements are made with a spectrophotometer.

2.5. Statistical Analysis

SPSS software (version 20) was used for statistical analysis. One-way analysis of variance (ANOVA) and Post-HOC LSD tests were used to evaluate the significance between the control and treatment groups. The obtained data were presented as mean and standard deviation.

3. Results

In the MTT and LDH tests, which measure cell viability, it was determined that the cell viability of the PC group was significantly reduced compared to the NC group (67.87%). However, cell viability in the treatment groups was 82.27%, 90.89% and 81.24%, respectively. In the LDH analysis, while the accumulation of LDH in the PC group was 235.55% compared to the NC group, it was 175.24%, 138.32% and 155.68% in the treatment groups, respectively. When all data were evaluated, when compared to the PC group in both tests, the group with the highest cell viability was the GG 200 µg/ml group. All treatment groups had statistical significance compared PC group in both tests ($p<0.05$, Figure 1).

After administration of glutamate toxicity, MDA and TOS levels were significantly higher in the PC group. SOD and TAS levels were significantly lower ($p<0.05$). MDA and TOS levels were significantly lower in the treatment groups. SOD and TAS levels were significantly higher than PC group ($p<0.05$). When the treatment groups were examined among themselves, the levels of TOS and MDA associated with oxidative stress were the lowest in cells treated with 200 µg/ml GG. TAS and SOD antioxidant levels were found to be higher in the 200 µg/ml GG group compared to the other treatment groups (Figure 2).

4. Discussion

Plants have been used in the treatment of various diseases for hundreds of years. Today, it continues to be a source for drugs used in modern medicine due to the bioactive molecules they contain [17]. In this study, it was determined by biochemical analyzes that GG water extract alleviated glutamate toxicity in primary neuron culture. Ancient Egypt, Greece, China, India, and Roman civilizations used GG as an expectorant and carminative. In pharmacological studies, it has been proven to have antioxidant, antimalarial, anti-inflammatory, antidiabetic, antiulcer and neuroprotective activity [2, 18]. Petramfar et al. in a double-blind randomized clinical trial, one group of PD patients received syrup prepared using GG twice a day for 6 months, while the other group of PD patients received only placebo syrup. Compared to the placebo group, they reported that tremor and daily activities showed significant improvement from the 4th month in PD patients using GG syrup, and their blood values and motor tests were better [19]. Karthikkeyan et al. determined that GG water extract increased cell survival, decreased mitochondrial stress by inhibiting MEK-ERK-1/2 hyper-phosphorylation, and prevented apoptosis in an in-vitro PD model created using retenone in the MR-32 cell line [20].

Studies have shown that GG contains flavonoids such as liquirtin, rhamnoliquirilin, liquiritigenin, prenyllicoflavone A, glucoliquiritin apioside, 1-metho-xyphaseolin, shinpterocarpin, shinflavanone, licopyranocoumarin, glisoflavone, licoarylcoumarrrhizain, GR, and important phenolic components such as isoangustone A, semilicoisoflavone B, licoriphenone and 1-methoxyficifolinol [21, 22]. GR, also known as glycyrrhizic acid, is the main active ingredient of GG root extract [22]. Since GR, which has antioxidant and anti-inflammatory properties, can cross the blood-brain barrier, it has been suggested as a drug target especially for the treatment of neurodegenerative diseases. Neuroprotective agents generally target a certain class of neurotransmitter receptors that mediate neurotoxic agents [23]. Lou et al. reported that GR reduced neuronal cell death in a kainic acid-induced toxicity model in the mouse hippocampus. Although it could not prevent epileptic seizures, they found that GR provided neuroprotection in the brain through anti-inflammatory and anti-excitotoxic effects [24]. Glutamate is the main excitatory neurotransmitter in the central nervous system (CNS) and is responsible for most physiological events in the CNS [25]. Excessive glutamate secretion accelerates oxidative stress by increasing intracellular calcium influx, reactive molecule increase and lipid peroxidation [26]. Cherng et al. reported that glycyrrhizic acid provides neuroprotection in a glutamate-induced rat primary neuron toxicity model, which is responsible for an important part of neurodegenerative diseases. They determined that the neuroprotective effect of glycyrrhizic acid was achieved by selectively inhibiting the binding of nuclear factor kappa B (NF-κB) to the target and the Ca^{2+} flux activated by glutamate via the NMDA receptor [27]. In this study, in accordance with the studies in the literature, glutamate toxicity created in rat primary neuron culture increased cell survival in MTT and LDH measurements depending on the dose. Other active ingredients are likely to contribute to this effect, as well as GR, which is the main component of GG. Liquiritin, an important flavonoid, was isolated from GG for the first time. Studies have shown that liquiritin has various pharmacological properties, including cardiovascular protective, pulmonary protective, hepatoprotective, skin protective, antitumor and neuroprotective effects [28]. Shi et al. determined that against SH-EP1 cell damage induced by hydrogen peroxide, liquiritin reduces oxidative stress-related damage by inhibiting the mitochondrial pathway of apoptosis and increasing the function of the antioxidant system [29]. Nakatani et al. reported in a study that liquiritin increased the proliferation of B65 neuroblastoma cells, reduced oxidative stress by increasing glucose-6-phosphate dehydrogenase against neurotoxic agents, and provided a neuroprotective effect [30]. Although its effects such as inhibition of anti-HIV, CYP3A4 and aryl hydrocarbon receptor antagonist have been reported, there is limited research on licopyranocoumarin. Fujimaki et al. reported that licopyranocoumarin provides neuroprotection by inhibiting LPC, GCR, MPP+-induced ROS production in the PD model created in the PC12D cell line [31]. Glabridin is one of the most studied licorice flavonoids and is a neuroprotective compound with antioxidant, anti-inflammatory, anticholinesterase properties. It has been shown to have positive effects on learning and memory in rats with Alzheimer's disease [32]. Yu et al. determined that glabridin ameliorated the damage caused by brain injuries caused by middle cerebral artery occlusion in rats and modulated multiple apoptosis-related pathways in staurosporine-induced rat cortical neurons in-vitro [33]. In this study, oxidative stress parameters MDA and TOS levels were lower in the GG extracts groups compared to the PC group. On the other hand, SOD and TAC levels, in which the antioxidant effect was investigated, increased in the GG extract applied groups. This is thought to be a synergistic result of the antioxidant effect of GR, liquiritin, licopyranocoumarin and glabridin, the main components of the GG extract, by reducing mitochondrial oxidative stress.

5. Conclusion

Glycyrrhiza glabra L. has components with pharmacological activity. In this study, we determined that GG significantly reduced glutamate toxicity in rat primary neuron cells. GG glutamate excitotoxicity and oxidative stress have a strong potential to protect from stress-related neurodegenerative diseases. However, these effects need to be supported by preclinical and clinical studies.

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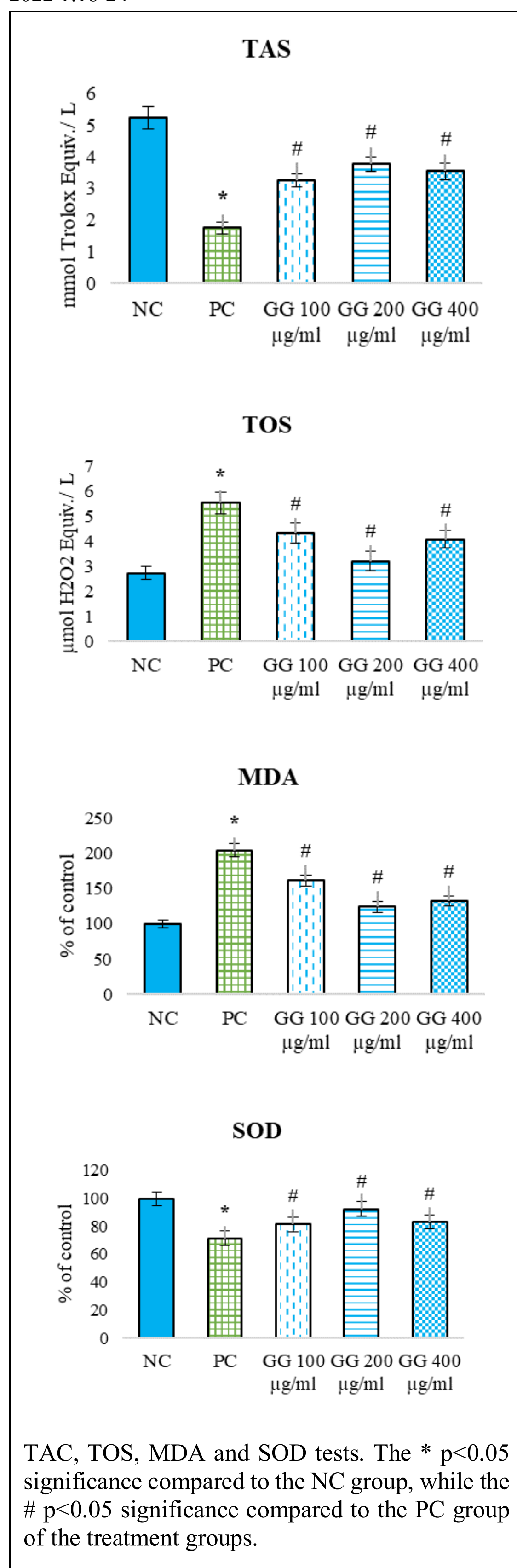

TAC, TOS, MDA and SOD tests. The * p<0.05 significance compared to the NC group, while the # p<0.05 significance compared to the PC group of the treatment groups.

Figure 2. Biochemical Analyzes

Conflict of Interest

The authors declare no conflict of interest.

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