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The protective effect of Chrysin against glutamate-induced oxidative stress and inflammation in SH-SY5Y cells

Adem Ahlatcı[1], Yener Yazğan[2], Kenan Yıldızhan[3]

[1]Department of Health Technicianship, Vocational School of Health Services, Van Yüzüncü Yıl University, Van, Turkiye
[2]Department of Biophysics, Faculty of Medicine, Kastamonu University, Kastamonu, Turkiye
[3]Department of Biophysics, Faculty of Medicine, Van Yüzüncü Yıl University, Van, Turkiye

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ABSTRACT

Aims: Chrysin is a flavonoid with economic value and medicinal effects commonly found in propolis, honey, and blue passion flowers. It has many pharmacological properties such as anticancer, antitumor, antioxidant, neurotrophic, and antidepressant. Glutamate plays an important role in brain functions; however, its high concentration in the central nervous system causes neurotoxic effects. This study aimed to investigate the effect of Chrysin on glutamate-induced cytotoxicity in SH-SY5Y neuroblastoma cells.

Methods: This study was conducted in 4 groups; control, Chrysin (50 μM), glutamate (10 mM), and glutamate+Chrysin. cell viability, total oxidant status (TOS), total antioxidant status (TAS), tumor necrosis factor alfa (TNF-α), and interleukin 1 β (IL-1β) levels in the cells were determined by ELISA kit.

Results: It is shown that glutamate application caused cytotoxicity in SH-SY5Y neuroblastoma cells and increased TOS, TNF-α, and IL-1β levels. However, in SH-SY5Y cells treated with Chrysin before glutamate incubation, TOS, TNF-α, and IL-1β levels decreased compared to the glutamate group, while TAS and cell viability levels increased.

Conclusion: Chrysin's antioxidant properties played a protective role in SH-SY5Y cells against glutamate-induced increased oxidative stress and inflammation.

Keywords: Anti-inflammatory, Chrysin, glutamate, oxidative stress, SH-SY5Y cells

INTRODUCTION

Chrysin is a 15-carbon skeleton polyphenolic molecule belonging to the flavone class.[1] Propolis, honey, and passion fruit (passiflora spp.) are the most significant natural sources. The oral bioavailability of Chrysin is low. It is rapidly excreted with faeces. Sulfate form in plasma can be detected. It has very low bioavailability as it is a substance with very low solubility, poorly absorbed, and rapidly metabolized.[2] Unlike other flavonoids, Chrysin has a chemical structure with hydroxyl groups (5,7-dihydroxy) on the A ring and no adducts on the B ring. The absence of oxygenation in the B and C rings is linked to the biological actions of Chrysin. The chemical structures of certain flavones determine their antioxidant action. Chrysin's antioxidant action is intimately linked to the carbonyl group on C-4 and the double bond between C-2 and C-3.[3] Chrysin is effective against obesity and cardiovascular diseases. It has many properties such as antiallergic, hepatoprotective, neuroprotective, anticancer, antidiabetic, antibacterial, antihypertensive, vasodilator, anxiolytic, antiviral, antiestrogenic, antiaging, anticonvulsant, antioxidant and anti-inflammatory effects. It increases mesenchymal stem cell proliferation.[4-6]

Glutamate is a neuroessential dicarboxylic amino acid found almost everywhere in the nervous system and involved in neuron cell metabolism. The studies conducted in the 1950s proved to be responsible for neuronal excitation in mammalian spinal cord cells; subsequent studies have shown that it functions as a neurotransmitter in the thalamus, hippocampus, cerebral cortex, primary sensory nucleus, and reticular formation. It is accepted that glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS) and is responsible for 1/3 of the excitatory synapses.[7] Glutamate is stored in synaptic vesicles, similar to other neurotransmitters. After passing into the synaptic gap, it is taken back by glial cells and converted to glutamine. Glutamine is transferred to neurons through carriers using active transport, where it is converted back to glutamate by glutaminase and stored in vesicles. In addition, glutamate can also be taken back by pre-post neurons. The cell is highly regulated in the release and reuptake of glutamates.[8] As a result of neurons' excessive activation, the calcium ion (Ca^{2+}) ratio inside the cell increases. For this situation to occur, both ionotropic and metabotropic glutamate

Corresponding Author: Yener Yazğan, yener8275@hotmail.com

receptors are needed. As a result of glutamate binding, the ionotropic NMDA receptor causes Ca^{2+} to enter the cell from the extracellular space. Since this situation causes the Ca^{2+} balance within the cell to deteriorate, cell death is triggered through mitochondrial membrane depolarization, caspase activation, and the formation of free radicals. The formation of free radicals is effective in excitotoxic cell death.[9]

Although glutamate plays an important role in brain functions, its high concentration in the CNS causes the neurotoxic effect of glutamate. The positive effects of phenolic components on neurotoxic effects are known. However, the effect of Chrysin, one of the important phenolic components, on glutamate excitotoxicity has not yet been fully determined. This study aimed to examine the effect of Chrysin on glutamate excitotoxicity in the SH-SY5Y neuroblastoma cell line.

METHODS

Ethics

The current study does not involve research conducted with human subjects or participants. Commercially purchased cell lines are not subject to ethics committee approval. All procedures were carried out in accordance with the ethical rules and the principles.

Cell Culture and Experimental Groups

SH-SY5Y cells are one of the most commonly used cell lines in neurotoxicity studies and were obtained from ATTC (American Type Culture Collection).[10] Cells were grown in Dulbecco's modified eagle medium (DMEM) cell culture medium containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin) in 75 cm^2 flasks at 37°C in a CO_2 incubator. For 24 hours, the cells were seeded in 75 cm^2 cell culture flasks. Glutamate and Chrysin were obtained from Sigma-Aldrich (St. Louis, MO, USA).

The study was conducted in 4 groups;

- **Control group:** No treatment was applied to this group,
- **Chrysin group:** 50 µM Chrysin was added and incubated for 24 hours.[11]
- **Glutamate group:** 10 mM glutamate was added and incubated for 24 hours.[12]
- **Glutamate+Chrysin group:** The SH-SY5Y cells were treated with Chrysin 50 µM 1 h prior to glutamate exposure and incubated for 24 hours.

Cell Viability Assay

The SH-SY5Y system, which was planted in a vial, was removed with trypsin EDTA after reaching sufficient stability. It was counted under an inverted microscope. The calculated cells were planted in 96-well plates with $7x10^3$ cells in each well. The CCK-8 (Abbkine, Cat#KTA1020) test, which gives color through mitochondrial enzymes, was used to evaluate cell viability after glutamate toxicity of Chrysin. Cell viability was measured at 450 nm using commercial ELISA kits on the BioTek ELx808™ instrument, following the instructions in the kit procedure. Experiments were repeated three times to determine the cell viability rate in the groups.

Measurement of Biochemical Parameters in SH-SY5Y Cells by ELISA Kits

Before starting the study, they were placed in separate sterile tubes for each group. The tubes were centrifuged at 1000 rpm for 20 minutes according to the kit procedure. After removal of the supernatants, cell pellets were suspended in PBS at a concentration of approximately 1x 10^6 cell/ml cells at pH: 7,4. Cells were lysed to separate them from elements resulting from repeated freeze-thaw cycles. The Bradford protein determination method was developed by taking advantage of the fact that the Coomassie Brilliant Blue G-250 dye molecule creates the blue colour of different intensities in protein solutions at different concentrations in the presence of protein and in an acidic environment.[13] Total antioxidant status (TAS), total oxidant status (TOS), tumor necrosis factor alfa (TNF-α), and interleukin 1 β (IL-1β) levels in the supernatants of SH-SY5Y cells were determined by ELISA kits (Bioassay Technology Lab. China). Measurements were made using ELISA kits BioTek ELx808™ device by the kit procedures.

Statistical Analysis

All data were presented as mean±standard deviation (SD). For statistical evaluation, one-way ANOVA (analysis of variance) was performed using the SPSS software for normally distributed data. The post-hoc Tukey test was conducted for all data that showed statistically significant differences. The results were expressed as mean±standard deviation (mean±SD). All probability test results were considered significantly different at $p<0.05$.

RESULTS

Effect of Chrysin on SH-SY5Y Cell Viability

In this study, the effect of Chrysin against glutamate toxicity was used to investigate cell viability in the study groups. For this purpose, the CCK-8 cell viability kit was employed. To examine the growth inhibitory effect of Chrysin, the anti-proliferative efficacy of Chrysin on SH-SY5Y cells was first assessed (**Figure 1**). It was observed that cell viability decreased significantly in the glutamate group compared to the control and Chrysin groups ($p<0.05$). It was observed that the cell viability level increased significantly in the cells in the glutamate+Chrysin group compared to the glutamate group ($p<0.05$).

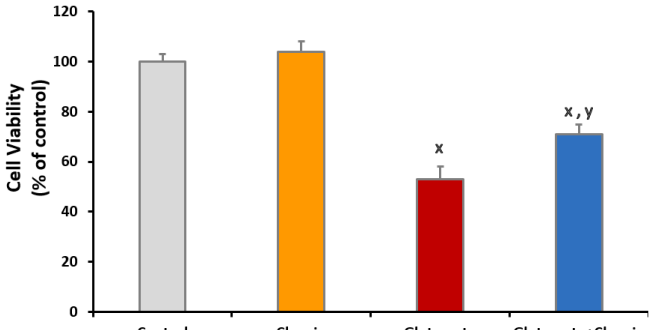

Figure 1. Effect of Chrysin on cell viability after glutamate-induced excitotoxicity in the SH-SY5Y cells. $^{x}p<0.05$ as compared to control and Chrysin groups, $^{y}p<0.05$ compared to glutamate group

Effect of Chrysin on TAS and TOS Lvels in SH-SY5Y Cells

Colourimetric TAS and TOS measurement commercial kits were used to evaluate the effects of Chrysin on oxidative stress after excitotoxicity caused by glutamate in cell lysates. According to TAS results, the application of 10 mM glutamate to SH-SY5Y cells showed a decrease in TAS level compared to the control and Chrysin groups ($p<0.05$; **Figure 2**). It was observed that the TAS level increased significantly in the cells

in the glutamate+Chrysin group compared to the glutamate group.

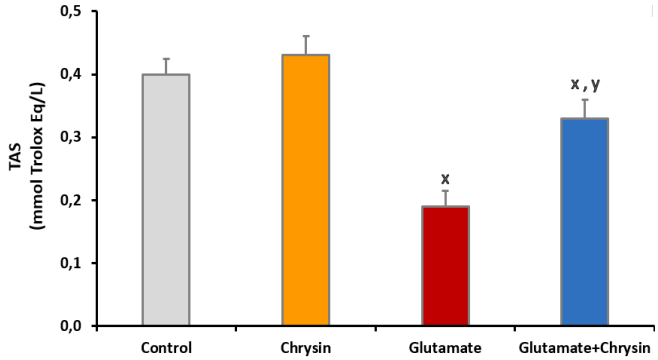

Figure 2. Effect of Chrysin on TAS level in SH-SY5Y cells after glutamate-induced cytotoxicity. $^{x}p<0.05$ as compared to control and Chrysin groups, $^{y}p<0.05$ compared to glutamate group

TAS: Total antioxidant status

According to TOS results, the application of 10 mM glutamate to SH-SY5Y cells significantly increased the TOS level compared to the control and Chrysin groups ($p<0.05$; **Figure 3**). There was no statistical difference between the control and Chrysin groups. It was observed that the TAS level increased in the cells in the glutamate+Chrysin group compared to the glutamate group, and the TOS level decreased significantly ($p<0.05$).

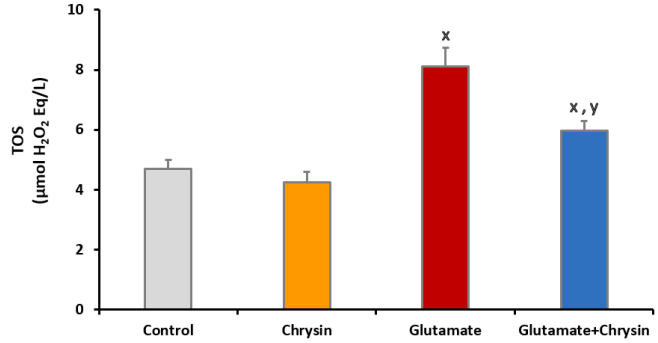

Figure 3. Effect of Chrysin on TOS level in SH-SY5Y cells after glutamate-induced cytotoxicity. $^{x}p<0.05$ as compared to control and Chrysin groups, $^{y}p<0.05$ compared to glutamate group

TOS: Total oxidant status

Effect of Chrysin on TNF-α and IL-1β Levels in SH-SY5Y Cells

TNF-α ELISA commercial kit was used to measure TNF-α values in Chrysin-applied and non-applied groups in SH-SY5Y cells in which glutamate excitotoxicity was induced. ELISA test results showed that TNF-α levels in the glutamate group increased statistically significantly compared to the control and Chrysin groups ($p<0.05$; **Figure 4**). On the other hand, a significant decrease in TNF-α levels was detected in the group given Chrysin together with glutamate compared to the glutamate group ($p<0.05$).

IL-1β ELISA commercial kit was used to evaluate the effect of Chrysin on IL-1β levels in SH-SY5Y cells with glutamate excitotoxicity. The test data obtained as a result of the application showed that IL-1β levels in the glutamate group increased significantly compared to the control and Chrysin groups ($p<0.05$; **Figure 5**). A significant decrease in IL-1β level was detected in cells treated with Chrysin along with glutamate compared to the glutamate group ($p<0.05$).

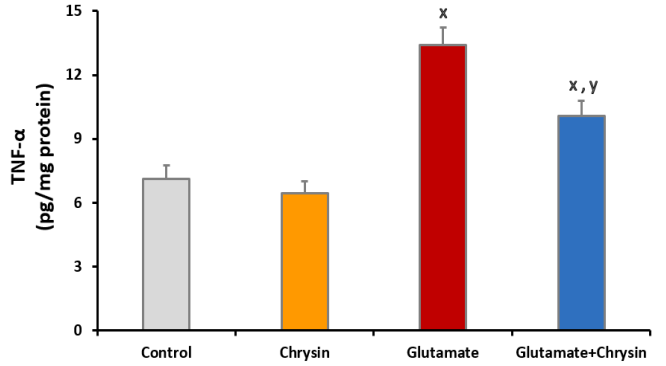

Figure 4. Effect of Chrysin on TNF-α level in SH-SY5Y cells after glutamate-induced cytotoxicity. $^{x}p<0.05$ as compared to control and Chrysin groups, $^{y}p<0.05$ compared to glutamate group

TNF-α: Tumor necrosis factor alfa

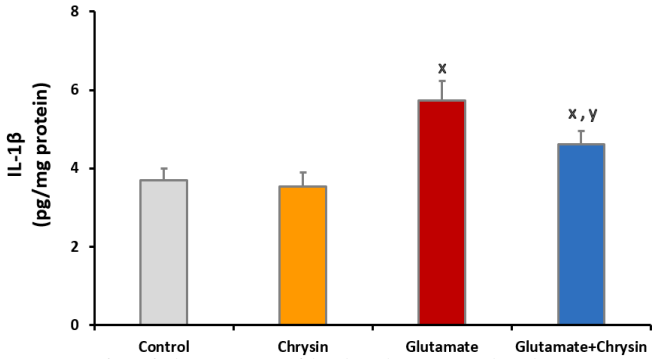

Figure 5. Effect of Chrysin on IL-1β level in glutamate-induced cytotoxicity in SH-SY5Y cells. $^{x}p<0.05$ as compared to control and Chrysin groups, $^{y}p<0.05$ compared to glutamate group

IL-1β: Interleukin 1 β

DISCUSSION

Glutamate has important functions as a major excitatory neurotransmitter in the mammalian brain.[14] However, excessive release of glutamate results in excitotoxicity. Excitotoxicity is an important factor in a variety of pathologies, from ischemic brain injury to neurodegenerations associated with many acute and chronic diseases.[15] Many studies have been conducted to understand the pathogenesis of these diseases. The basic mechanism is excitotoxicity caused by excessive glutamate stimulation. Various studies have been conducted to prevent pathologies caused by glutamate-induced excitotoxicity.[15-17] Although many substances with anti-inflammatory, antioxidant, and antiproliferative properties have been used in studies to prevent excitotoxicity through various mechanisms, no study has been found in the literature on the effect of Chrysin on glutamate excitotoxicity. Studies have shown that Chrysin exhibits a wide range of bioactivities, such as antitumor, antiulcer, antimicrobial, and antiproliferative effects on cancer cells.[18] One of the anticancer, antiulcerative and antiproliferative effects of Chrysin is to protect the cell from apoptosis by reducing oxidative stress. In our study, the effect of Chrysin pre-treatment against glutamate-dependent toxicity induced in vitro was examined.

It was demonstrated that cyclophosphamide caused cell toxicity in SH-SY5Y cells and that antioxidant treatments suppressed cell death in a study assessing the potential therapeutic effects of ferulic acid, quercetin, Chrysin, and caffeic acid against cyclophosphamide-induced toxicity in SH-SY5Y neuronal cells. By lowering lipid peroxidation levels,

downregulating the expression of Bax, cytochrome c, and Cas-3, and upregulating the expression of the anti-apoptotic gene Bcl-2, Chrysin protected SH-SY5Y cells.[19] In another study on Chrysin, it was shown that Chrysin suppressed diclofenac-induced apoptosis and oxidative damage in HT-29 cells and reduced cell death. Diclofenac-induced cell death caused an increase in ROS, malondialdehyde levels, and lactate dehydrogenase release, while it led to a decrease in total antioxidant and catalase activity. It was reported that pretreatment with Chrysin reversed these effects. Additionally, the expression levels of p53, Cas-3, Cas-8, Bax, and cytochrome c increased in the diclofenac-treated group but decreased following treatment with Chrysin.[20] In our study, it was determined that pretreatment with Chrysin increased cell viability and reduced cell death against glutamate-induced cytotoxicity in SH-SY5Y neuroblastoma cells (Figure 1). In line with the literature, our study results showed that high-dose glutamate exposure reduces cell viability by triggering intracellular oxidative stress-induced cascade pathways, whereas Chrysin' prevents this damage mechanism thanks to its antioxidant properties.

In a study on glutamate excitotoxicity in SH-SY5Y neuroblastoma cells, it was shown that TAS levels decreased while TOS levels increased.[21] In another study on glutamate excitotoxicity using primary rat cortical neurons, it was found that glutamate reduced TAS levels and increased TOS levels.[22] Similarly, in our study, TAS levels decreased, whereas TOS levels increased in the glutamate group (**Figure 2, 3**). In our study, Chrysin increased cell viability and TAS levels and decreased TOS levels by showing antioxidant properties in SH-SY5Y cells. In this context, we can also say that Chrysin has a protective effect against glutamate-induced neurotoxicity.

In neuroinflammatory diseases, the neuronal system and functions in the CNS are disrupted. Neuroinflammation is a protective mechanism aimed at repairing damaged glial cells and neuronal cells in the CNS. Unlike other cells, once neurons are damaged or degenerate, they cannot be repaired or regenerated in the CNS. Neuroinflammation is an important protective response for the brain, but excessive inflammatory responses are dangerous and can hinder neuronal regeneration. Microglia and astrocytes are influenced and altered by peripheral inflammatory conditions, thereby affecting neuroinflammation and neurodegeneration.[23]

A proinflammatory cytokine, TNF-α plays crucial roles in the central nervous system's homeostatic and pathological processes. Significant amounts of TNF-α are released by microglia under pathological conditions; this production is an essential component of the neuroinflammatory response associated with a number of neurological diseases. Furthermore, TNF-α can increase glutamate-induced cytotoxicity in two complementary ways: first, by indirectly blocking astrocyte glutamate transport processes; and second, by simultaneously decreasing the number of inhibitory GABA receptors and quickly increasing the surface expression of Ca^{2+}-permeable AMPA and NMDA receptors in neurons. Thus, the overall impact of TNF-α is to increase the synaptic excitatory/inhibitory ratio by shifting the balance between excitation and inhibition.[24]

There is a strong relationship between oxidative stress and cytokines, especially TNF-α.[25] Increased TNF-α level leads to oxidative stress. Previous studies have shown the existence of a parallel relationship between TNF-α and the reduction of oxidative stress during the healing process.[26] Therefore, we examined the relationship between the reduction of oxidative stress mediated by Chrysin and TNF-α. Proinflammatory cytokines are both key mediators and key mediators of neuroinflammation, and they have an important role in responding to neuronal damage.[27-29] Strong evidence indicates that proinflammatory cytokines stimulated by glutamate are involved in the progression and pathophysiology of neuronal diseases.[30] We observed that TNF-α expression significantly increased after glutamate application to SH-SY5Y cells and that Chrysin application prevented these neuroinflammatory events. In this context, Chrysin exhibits antioxidant and anti-inflammatory properties. We can also say that they have a protective effect against glutamate-induced neurotoxicity.

The first studies showing that TNF-α can potentiate excitotoxicity were conducted in human neuron cultures.[31] In a study investigating the effects of Chrysin against hepatotoxicity induced by high-dose paracetamol, it was observed that paracetamol increased lipid peroxidation and liver enzyme activities in rats while reducing antioxidant enzyme activities. Paracetamol also triggered an inflammatory response by raising the levels of TNF-α and IL-1β.[32] In a study on Chrysin, known as Chinese propolis, its effects against tunicamycin-induced neuronal cell death were evaluated in SH-SY5Y cells. Chrysin inhibited tunicamycin-induced cell death, caspase-3 activation, and its effects on mitochondria in a concentration-dependent manner.[33] In a study aimed at investigating the potential protective effects of Chrysin on oxidative status and the histological changes in liver and kidney tissues induced by carbon tetrachloride (CCl4) in rats, it was observed that CCl4 increased the levels of MDA, serum TNF-α, AST, ALT, urea, and creatinine, and the addition of Chrysin reversed these results.[34] In another study, he protective properties of Chrysin against cyclophosphamide-induced cardiotoxicity in rats were investigated. The results showed that cyclophosphamide significantly increased cardiac MDA, TNF-α, IL-1β, and IL-6 levels. And also, cardiac antioxidant enzyme activities such as superoxide dismutase (SOD) and catalase (CAT), as well as glutathione (GSH) levels, were suppressed. Treatment with Chrysin significantly restored these altered biochemical and antioxidant properties. Consequently, these findings suggest that Chrysin offers cardioprotective potential against cyclophosphamide-induced cardiotoxicity, which may be attributed to its antioxidant and anti-inflammatory properties.[35] In our study, Chrysin exhibited a protective effect in SH-SY5Y neuroblastoma cells by reducing the levels of TNF-α and interleukin-1β inflammation markers, which increased as a result of glutamate excitotoxicity (**Figure 4, 5**).

CONCLUSION

As a result, this study concluded that Chrysin increased the proliferation of SH-SY5Y neuroblastoma cells. The research findings indicate that an appropriate dose of Chrysin enhances cellular resistance to glutamate-induced cytotoxicity in these cells. We propose that glutamate's harmful effects are mediated through the activation of oxidative stress pathways. Consequently, it is suggested that Chrysin at the right dosage may offer protection against glutamate toxicity in central nervous system disorders and serve as a valuable therapeutic

agent for conditions associated with neuronal damage. Also, it was found in the study that Chrysin affects the SH-SY5Y cells. Nonetheless, additional in vitro and in vivo studies are required to explore the potential mechanisms by which Chrysin exerts its effects on glutamate toxicity.

ETHICAL DECLARATIONS

Ethics Committee Approval

Ethics committee approval is not required for cell culture studies.

Informed Consent

Because the study has no study with human and human participants, no written informed consent form was obtained.

Referee Evaluation Process

Externally peer-reviewed.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Financial Disclosure

The authors declared that this study has received no financial support.

Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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