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Etanercept Protects Neurons Against Glutamate-Induced Neurotoxicity: An In Vitro Study

Etanersept glutamat ile indüklenen nörotoksisiteye karşı nöronları korur: Bir in vitro çalışma

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ABSTRACT

Current study was designed to investigate the protective effects of etanercept in glutamate excitotoxicity in rat neuronal culture through anti-inflammatory and anti-oxidant mechanisms. Rat cortical neurons were exposed to glutamate and to assess the effect of etanercept in glutamate toxicity, etanercept was applied at various doses (0.1, 0.5, 1, 10 µg/ml). Then we examined the changes in neuronal cell viability, oxidative stress and inflammation. Etanercept preserved cultured cells from glutamate excitotoxicity. Our cell viability analysis (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and lactate dehydrogenase) revealed that etanercept markedly increased the viability ratio of neurons injured by glutamate. In addition, the potential antioxidant property of etanercept was evaluated via the examination of oxidative stress parameters, such as MDA and TOS, and antioxidant parameters measured as TAS and SOD. Moreover, TNF-α levels were measured to evaluate anti-inflammatory effects of etanercept. Obtained data proved that etanercept increases the activity of the antioxidative parameters while decreased oxidative parameters and inflammation. The current study demonstrated that etanercept strongly prevents glutamate- induced neuronal cell death. This study is the first to demonstrate a potential protective effect of etanercept in neurons exposed to glutamate excitotoxicity and opens new doors on the therapeutic potential of etanercept.

Keywords: Etanercept, glutamate, in vitro, neuroprotection, oxidative stress

ÖZ

Mevcut çalışma, etanerseptin glutamat eksitotoksisitesinde koruyucu etkilerini, anti-inflamatuar ve anti-oksidan mekanizmalar yoluyla sıçan nöron kültüründe araştırmak için tasarlanmıştır. Sıçan kortikal nöronları glutamata maruz bırakıldı ve ardından etanerseptin glutamat toksisitesindeki etkisini değerlendirmek için etanersept çeşitli dozlarda (0.1, 0.5, 1, 10 µg/ml) uygulandı. Daha sonra nöronal hücre canlılığı, oksidatif stres ve inflamatuvar değişiklikleri inceledik. Etanerseptin, hücreleri glutamat eksitotoksisitesinden korudu. Hücre canlılığı analizlerimiz (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide ve laktat dehidrogenaz), etanersept'in glutamat tarafından hasarlanan nöronların canlılık oranını belirgin şekilde arttırdığını ortaya koydu. Ayrıca, etanerseptin potansiyel antioksidan özellikleri MDA ve TOS gibi oksidatif stres parametrelerinin incelenmesi ve TAS ve SOD olarak ölçülen antioksidan parametreleri ile değerlendirildi. Ayrıca etanerseptin antiinflamatuar etkilerini değerlendirmek için TNF-α seviyeleri ölçüldü. Elde edilen veriler, etanercept'in inflamasyonu ve oksidatif parametreleri azaltırken antioksidatif parametrelerini arttırdığını kanıtladı. Bu çalışma, etanerseptin glutamat kaynaklı nöronal hücre ölümünü güçlü bir şekilde önlediğini göstermiştir. Bu çalışma, glutamat eksitotoksisitesine maruz kalan nöronlarda etanerseptin potansiyel koruyucu etkisini gösteren ilk çalışmadır ve etanerseptin terapötik potansiyeli hakkında yeni kapılar açmaktadır.

Anahtar Kelimeler: Etanersept, glutamat, in vitro, nöroproteksiyon, oksidatif stress

INTRODUCTION

L-glutamate is one of the most crucial excitatory neurotransmitters of the central nervous system (CNS) and plays a prominent role in various physiological processes including synaptic plasticity, learning, memory, and other cognitive functions.[1-3] Although glutamate (Glut) takes part in CNS functions, it is neurotoxic at high concentrations in the CNS.[4] Excitotoxicity is induced by overstimulation of the Glut receptor due to excessive Glut release followed by increased Ca^{2+} entry. As a result of the increase in intracellular Ca^{2+} levels, mitochondrial dysfunction, protease activation, and reactive oxygen species (ROS) occur in parallel with the increase in neuronal cell death.[5,6] Oxidative stress is an important factor triggering neuronal cell death in neuropathological processes. The increase in glutathione causes oxidative stress by inhibiting glutathione synthesis and promoting excessive ROS.[4,7,8] Glut toxicity greatly participates in numerous neurodegenerative diseases, such as amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, Huntington's disease, and epilepsy.[9-11] Therefore, the protection of neuronal cells against Glut-induced excitotoxicity could be an effective therapeutic approach against neurodegenerative diseases.[4] Among the models used to induce experimental cell damage, there are studies using excitotoxins such as Glut as well as hyperacidity and hypoxia. Hypoglycemia, hypoxia, endotoxicity, and mechanical damage trigger impaired release, reuptake, and metabolism of Glut which eventually lead to cell damage and death.[12-14]

Etanercept is a biological medicinal product used to treat autoimmune diseases by interacting with tumor necrosis factor-alpha (TNF-α), an inflammatory cytokine. Etanercept unites with TNF-α and blocks TNF-α receptors' activity. Thereby, TNF-α activity decreases, and the inflammatory response is suppressed.[15-17] It has been shown that etanercept may have neuroprotective effects in traumatic brain injury and streptozocin-induced dementia.[18,19] In line with this information, it was intended to reveal the protective role of etanercept in Glut-induced neurotoxicity by cell viability test and molecular mechanisms.

MATERIAL AND METHODS

Primary Neuron Culture

Etanercept and glutamate were purchased from Sigma-Aldrich Darmstadt, Germany. This study was performed by consent of the Ethical Committee- Ataturk University Animal Experiments Local Ethics Committee (November 5, 2021-2100304005/9-23 0). In this study, newborn Sprague–Dawley rats that had not completed 24 hours were used to obtain cortex neurons. After the pups were decapitated, the removed cortices were transferred to 5 mL of Hanks' Balanced Salt solution, and macro fragmentation was performed with the help of a scalpel, and then micro fragmentation was performed with 0.25% Trypsin-et hylenediaminetetraacetic acid. Neurons were centrifuged at 1200 rpm for 5 minutes. Then, cellular medium 88% neurobasal medium, (Gibco MONTANA UNITED STATES), 10% fetal bovine solution, (Gibco), 2% B27 (Supplement, Thermo Fisher, Germany), 0.1% antibiotic (Penicillin–Streptomycin), and amphotericin B (Thermo Fisher) were supplemented. They were then incubated for 10 days at 5% CO_2 and 37°C. The medium was changed every 3 days.

To induce toxic damage, primary neurons were exposed to Glut at a concentration of 10^{-5}M for 5 minutes. Then, to assess the role of etanercept in Glut toxicity, etanercept was applied at various doses in separate wells (0.1 µg/mL, 0.5 µg/mL, 1 µg/mL, and 10 µg/mL) and incubated for 24 hours. Then, cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoli um bromide, yellow tetrazole, (MTT) analysis method.

Cell Viability Tests

The possible cytotoxic effect of etanercept on primary neuron cells with Glut toxicity was measured with the MTT kit in accordance with the manufacturer's instructions (Sigma, Mo, USA). Ten percent concentration of stock solution (MTT) was added to 96 well-plates. After incubation, 100 µL of dimethyl sulfoxide was added. Optical density was evaluated at 570 nm (BioTek Instruments California United States). Lactate dehydrogenase (LDH) activity was also evaluated with LDH assay kit (LDH assay kit, Elabscience Texas United States) consistent with kit procedure. Optical density was evaluated at 450 nm.

Biochemical Analysis

Cell media were collected 24 hours after the toxicity application, and measurements were made in accordance with the manufacturer's instructions to evaluate the total oxidant status (TOS), total antioxidant status (TAS) (Rel Assay Diagnostics, Gaziantep, Turkey), superoxide dismutase (SOD), malondialdehyde (MDA), and TNF-α (Elabscience).[6]

Statistical Analysis

Statistics were conducted by 1one-way analysis of variance with post hoc Tukey's test (IBM SPSS 20) ($P < .05$). Data were presented as mean ± standard deviation.

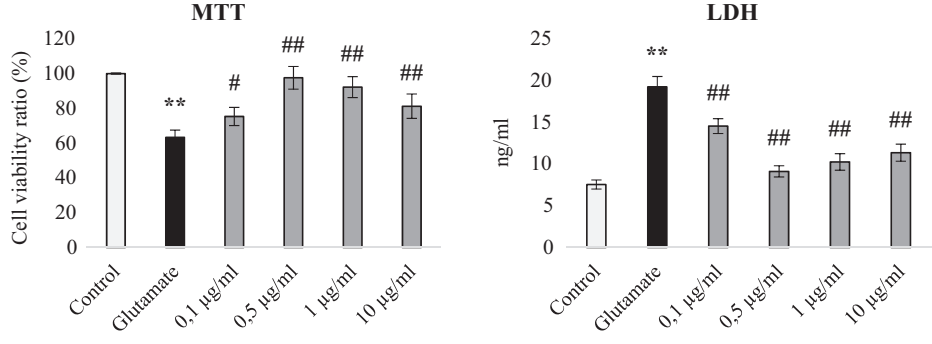

Figure 1. Effects of etanercept on the cell viability (MTT and LDH). Data are expressed as the mean ± SD. $^{**}P < .001$ vs. control group, $^{\#}P < .05$ vs. glutamate group, $^{\#\#}P < .001$ vs. glutamate group. MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase; SD, standard deviation.

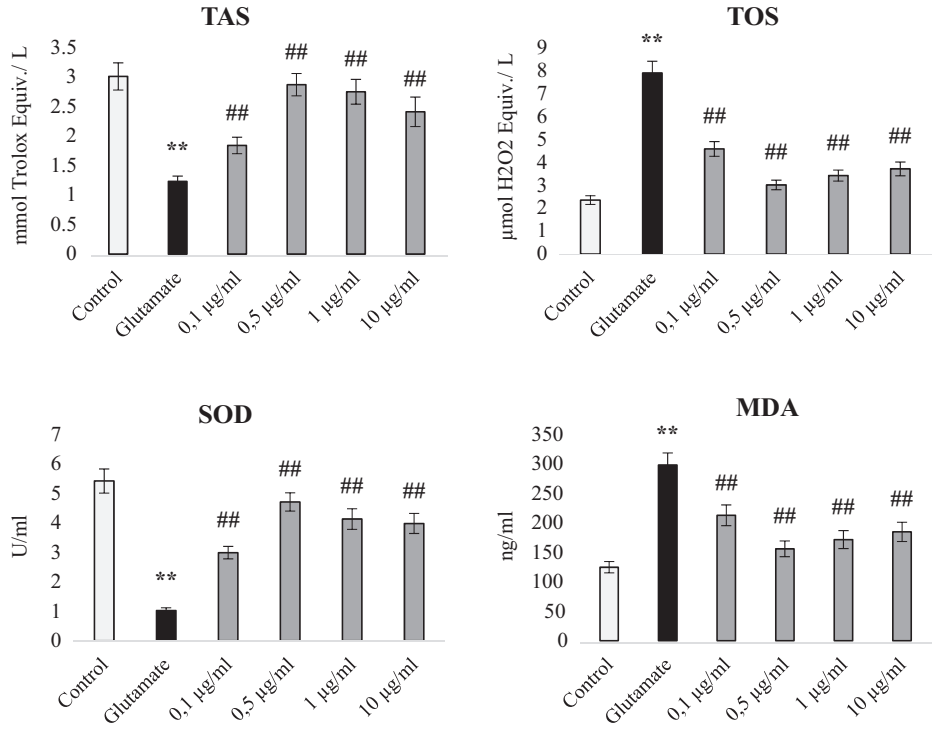

Figure 2. Effects of etanercept on the oxidative stress parameters (TAS, TOS, SOD, and MDA). Data are expressed as the mean ± SD. $^{**}P < .001$ vs. control group, $^{\#\#}P < .001$ vs. glutamate group. TAS, total antioxidant status; TOS, total oxidant status; SOD, superoxide dismutase; MDA, malondialdehyde; SD, standard deviation.

RESULTS

Effect of Etanercept on Viability of Neuronal Cells

The 10^{-5}M Glut led to a marked decline in viable cell rates in MTT analysis and increased LDH leakage. It was shown that etanercept provided a marked increase in neuronal proliferation in comparison to the Glut group ($P < .001$). Obtained data showed that 0.1 µg/mL, 0.5 µg/mL, 1 µg/mL, and 10 µg/mL etanercept markedly increased cell viability rate as compared to the Glut group ($P < .05$). In addition, etanercept notably decreased the LDH levels in comparison to Glut ($P < .001$) (Figure 1).

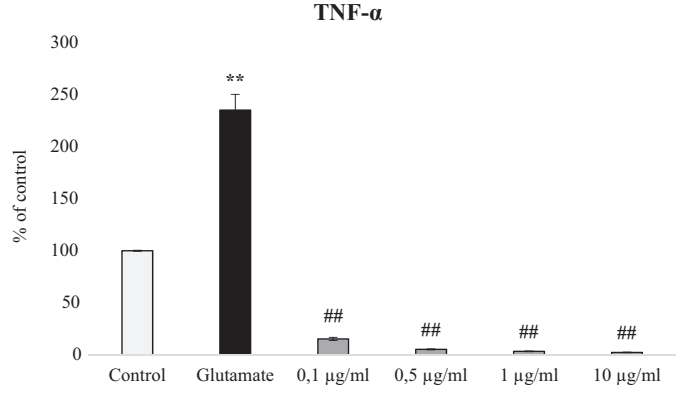

Figure 3. Effects of etanercept on the TNF-α levels. Data are expressed as the mean ± SD. $^{**}P < .001$ vs. control group, $^{\#\#}P < .001$ vs glutamate group. TNF-α, tumor necrosis factor-alpha; SD, standard deviation.

Effect of Etanercept on Glutamate-Induced Oxidative Stress in Neurons

Oxidative stress results were shown in Figure 2. The TAS and SOD activity were considerably decreased ($P < .001$) in Glut group. The TOS and MDA levels were markedly elevated ($P < .001$) in comparison with control. The TAS and SOD activity in etanercept groups were substantially enhanced when compared to the Glut group ($P < .001$), while TOS and MDA levels were markedly ($P < .001$) decreased than the Glut group.

Effect of Etanercept on Tumor Necrosis Factor-α Expression in Neuronal Cells

There was a marked elevation in the level of TNF-α, as a proinflammatory cytokine, in the Glut-treated group. The TNF-α concentrations are shown in Figure 3. It was found that TNF-α concentrations were notably elevated in the Glut-treated group as compared to the control group ($P < .001$). Etanercept markedly decreased TNF-α levels ($P < .001$) in comparison with the Glut group.

DISCUSSION

We have shown that etanercept preserved cortical neurons against Glut-induced cell death. We also observed that the suppression of oxidative stress and suppression of inflammation via blocking TNF-α by etanercept may be responsible for its neuroprotective effect on Glut-induced excitotoxicity in cortical neurons.

Glutamate is one of the most common neurotransmitters in the CNS, and Glut-related excitoxicity is main supporter of neuroinjury especially in CNS-related pathological diseases. Glut-induced neuroinjury is intervened by both excitotoxicity and oxidative stress.[20,21] Glut provokes the depolarization of the mitochondrial

membrane that sparks off changes in the mitochondrial membrane potential.

Mitochondrial activity, which plays a very important role in cell apoptosis, can be evaluated by the MTT assay.[22] In this study, treatment of etanercept markedly enhanced cell survival and diminished the inhibition of proliferation implying that etanercept might efficiently preserve mitochondrial form. Etanercept counteracted the impact of glutamate on neuronal cell survival.

A typical signal that indicates cell function is LDH activity/leakage. Lactate dehydrogenase is an enzyme found in all cells, and its amount increases in cell destruction. The greater the LDH released into the extracellular space, the greater the destruction in the cells.[23] Our study showed that etanercept reduced LDH release of neuron cells.

Moreover, Glut leads to increased intracellular oxidation and ROS generation resulting in apoptotic neuronal death.[24] Besides, it restricts glutathione formation by inhibiting Glut/cystine function giving rise to intracellular free radicals and cellular toxicity. Increased ROS trigger cell death by causing lipid peroxidation in the neuronal cell membrane.[25-28] Hasturk et al[29] previously showed that early administration of etanercept after spinal cord injury can significantly reduce neuronal damage by decreasing serum TNF-α levels, while increasing the levels of antioxidative enzymes, including SOD and CAT, in the subacute and acute stages.

Our data showed that etanercept has a marked function in the suppression of oxidative stress in neurons. When the amount of TAS and activity of SOD was measured, etanercept tends to increase the antioxidant capacity of cortical neuronal cultures. On the other hand, etanercept markedly suppressed the oxidative stress status which was manifested by decreased TOS and ROS levels in cortical cultures. These results imply that etanercept significantly reduced oxidative stress and increased antioxidant activity, and the activation of the antioxidant mechanism by etanercept occurs in the neuron cells.

Along with the glutamate, proinflammatory cytokines are known to regulate deterioration of the brain, reflected by neurological deterioration. In a previous study, a significant increase was observed in neuronal apoptosis and the presence of TNF-α in neurons and glial cells after neonatal exposure to glutamate; this result was in accordance with those obtained in other in vitro models of excitotoxicity.[30] It can be interpreted that the rapid increase in TNF-α expression in the damaged brain contributes to initiating cascades associated with neuronal apoptosis and neurological disorders.[31] When the functional importance of glut and its toxicity potential are considered together, a tight control mechanism over its release and reuptake is a necessity. The fact that both of these functions can also be controlled via TNF-α provides a solid therapeutic basis in neurodegenerative diseases for treatments based on reducing increased cerebral levels of this cytokine.[32] Increased TNF-α damages the CNS by increasing the level of Glut in the synaptic cleft and causing the neuronal death. Tumor necrosis factor-alpha is the first endogenous mediator reported to simultaneously affect cerebral levels of extracellular Glut by both increasing its release and decreasing its reuptake.[33,34] In a previous study, it was reported that intra-amygdala infusion of TNF-α increased Glut levels in the same region of the brain.[35] Similarly, etanercept has been reported to reduce brain Glut levels in different in vivo models.[36-38] In our study, it was shown that etanercept administration significantly decreased TNF-α levels compared to the Glut group.

In conclusion, etanercept, as a TNF-α inhibitor, has neuroprotective effects in primary neuron culture against Glut toxicity. It is thought that etanercept exerts this effect by increasing the antioxidant properties of cells while decreasing the oxidant capacity and TNF-α levels. Considering these effects of etanercept, it may serve as a therapeutic agent against Glut-induced neuronal death.

Ethics Committee Approval: Ethical committee approval was received from the Ethics Committee of Atatürk University Animal Experiments Local Ethics Committee (Date: 5.11.2021, Decision No: 21003 04005/9-23 0).

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