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# Nicorandil mitigates glutamate excitotoxicity in primary cultured neurons

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**Abstract**

Excitotoxicity, caused by the excessive release of glutamate, leads to the activation of the apoptotic process, making it a crucial factor in age-related neurodegenerative diseases. The aim of this study was to investigate the potential of nicorandil to prevent glutamate excitotoxicity and reduce oxidative stress in the brain by analyzing the effects of nicorandil on primary cortex neurons. The study used primary neuron cultures from newborn Sprague-Dawley rats to examine the impact of nicorandil on cell viability, Superoxide Dismutase, Catalase, Glutathione activity, Malondialdehyde levels, total antioxidant capacity, and total antioxidant status of neurons subjected to glutamate-induced excitotoxicity. Nicorandil at varying concentrations was introduced in the culture to assess its protective effects on the neurons. The results showed that nicorandil significantly improved cell viability and total antioxidant capacity levels and reduced total antioxidant status values in a concentration-dependent manner. These findings indicate that nicorandil effectively prevented glutamate excitotoxicity by reducing oxidative stress. The study suggests that nicorandil holds the potential for treating neurodegenerative diseases caused by glutamate excitotoxicity. This study is the first to report the potential of nicorandil to inhibit oxidative stress and prevent glutamate excitotoxicity in primary neurons, providing a basis for further exploration of the clinical application of nicorandil in neurodegenerative diseases.

**Keywords:** Glutamate, nicorandil, oxidative stress, primary neuron culture

## Introduction

As life expectancy has continued to increase from one century to the next, there has been a corresponding increase in the occurrence of age-related neurodegenerative diseases such as Alzheimer's, Parkinson's, and cerebral ischemia. This has led to these conditions becoming a significant worldwide health concern [1]. Research from the past century has shown that several free amino acids, such as glutamate, cysteine and aspartate, have a stimulating effect on the hippocampus and cerebral cortex and play a crucial role in memory and advanced cognitive processes by doing so [2].

Abnormal alterations in the physiological state of the neuronal excitatory system can lead to several neurological disorders. Glutamate is one of the primary excitatory neurotransmitters found in the brain and spinal cord. Plasticity and basal excitation play crucial roles in synaptic transmission. However, an excessive release of glutamate or prolonged exposure to it can cause overstimulation of glutamate receptors [3]. This overstimulation leads to an increase in intracellular calcium influx, which in turn raises mitochondrial membrane permeability and triggers the production of reactive oxygen species (ROS) [4]. An increase in ROS makes DNA, cellular lipids, and proteins susceptible to oxidative damage. This mitochondrial dysfunction causes a drop in energy production, which leads to the discharge of proapoptotic cytochrome c into the cytoplasm and the activation of apoptosis.

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Hence, reducing oxidative stress holds great promise as a means of mitigating the impact of glutamate neurotoxicity [5].

Nicorandil (N-[2-(Nitro-oxy)ethyl]-3-pyridine carboxamide) is a medication used to treat angina. It works by opening ATP-sensitive potassium channels, releasing nitric oxide (NO) into the environment, and promoting balanced dilatation of veins and arteries. By acting on vascular smooth muscle cells via cGMP and increasing NO, it causes vasodilation. Nicorandil also has the ability to close L-type voltage-dependent calcium channels and act on K+ ATP channels [6]. Its antioxidant properties are demonstrated by its ability to reduce oxidative stress by opening K+ ATP channels [7].

Despite the numerous studies on nicorandil, there is a lack of research on its ability to prevent neuronal damage caused by glutamate excitotoxicity. The rationale behind this study was to investigate the potential of nicorandil to prevent glutamate excitotoxicity by inhibiting oxidative stress, as it has been suggested that its efficacy on mitochondrial KATP channels and NADPH may play a role in this mechanism.

## Material and Methods

### Cell culture

This investigation was performed with the endorsement of the Atatürk University Local Ethics Committee for Animal Experiments (75296309-050.01.04-2100303998). The study employed newborn Sprague-Dawley rats that were less than 24 hours old to harvest cortex neurons. To accomplish this, the pups were decapitated, and the cortex was extrac ted and divided with a scalpel and 0.25% Trypsin-ethylenediaminetetraacetic acid. The neurons were then centrifuged and cultured in a cell medium consisting of 88% neurobasal medium, 10% fetal bovine solution, 2% B27 supplement, 0.1% antibiotic (Penicillin-Streptomycin), and amphotericin B. These primary neurons were then incubated for 10 days at a temperature of 37°C and a $CO_2$ concentration of 5%, with the medium being replaced every 3 days. After that, to cause toxic harm, the neurons were subjected to glutamate at a concentration of 10−5 M for a duration of 5 minutes. The effect of nicorandil was subsequently assessed by administering various dosages (10 µM, 100 µM, 500 µM, and 1000 µM) in different wells and incubating for 24 hours.

### Biochemical analysis

The cell viability was analyzed using the Diphenyltetrazolium Bromide (MTT) test. At the end of the experiment, 20 microliters (0.5 mg/ml) of MTT solution were added to all wells and incubated for 4 hours. Afterward, the medium was removed, and the MTT solution had transformed into formazan crystals within the mitochondria of living cells. To dissolve the formazan crystals, 100 microliters of DMSO were added. The optical densities of the wells were then measured with a spectrophotometer (EPOCH Take 3 Plate, BioTek) at 570 nm [8].

The analysis of total antioxidant capacity (TAC) is based on the principle of inhibiting the formation of the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) cation. At the end of the cell culture study, the medium is collected and ELISA kit procedure (Rel Assay Diagnostics, Gaziantep, Türkiye) is performed on it. The samples obtained at the end of the procedure give absorbance readings at 660 nm on the spectrophotometer (EPOCH Take 3 Plate, BioTek). The TAC value is calculated using the formula Trolox Equiv/mmol L-1 [9].

In the total antioxidant status analysis (TOS), the amount of oxidant is measured by using an ELISA kit (Rel Assay Diagnostics, Gaziantep, Türkiye) with a spectrophotometer. The ELISA kit procedure is applied to the media collected at the end of the cell culture studies. Measurements are taken with a spectrophotometer (EPOCH Take 3 Plate, BioTek) at 530 nm. The TOS value is calculated using the formula H2O2 equivalent/mmol L-1 [10].

The levels of Glutathione (GSH), Catalase (CAT), Malondialdehyde (MDA), and the activity of Superoxide Dismutase (SOD) in the cell media collected from the experimental groups were measured using an ELISA kit (Elabscience, USA) based on the Competitive-ELISA principle. After the ELISA kit procedure was completed, the optical density was measured at 450 nm with the use of a spectrophotometer (EPOCH Take 3 Plate, BioTek) [11].

### Statistical analyses

For data analysis, we utilized a statistical method called one-way analysis of variance (ANOVA) along with post hoc Tukey's test using IBM SPSS version 22.0 software [11,12]. In this study, we considered p-values less than 0.05 ($p<0.05$) as statistically significant, indicating that the observed results were unlikely due to chance. The data were presented as mean±standard deviation (SD), which allowed us to show the average value for each group along with the variation or spread of the data around the mean.

## Results

### The ımpact of nicorandil on the viability and proliferation of neuronal cells

The presence of 10−5 M glutamate resulted in a significant decrease in the number of viable cells, as demonstrated by MTT analysis, and also caused an increase in LDH leakage. However, the use of etanercept was found to promote a considerable increase in the proliferation of neuronal cells when compared to the group exposed to glutamate ($p<0.001$). Furthermore, the findings indicated that the application of nicorandil at concentrations of 10 µM, 100 µM, 500 µM, and 1000 µM resulted in a marked increase in cell viability rates when compared to the glutamate group ($p<0.05$). Additionally, nicorandil was found to significantly reduce LDH levels when compared to glutamate ($p<0.05$), as shown in Figure 1.

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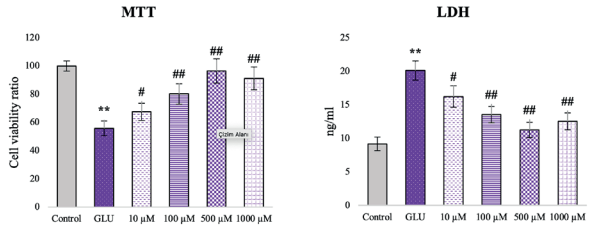


**Figure 1.** Effects of nicorandil on the cell viability (MTT and LDH). Data are expressed as the means±SD. ** p<0.001 vs control group, # p<0.05 vs GLU group, ## p<0.001 vs GLU group

### The ımpact of nicorandil on glutamate-ınduced oxidative stress in neuronal cells

The study presented the results of the oxidative stress analysis, which were depicted in Figure 2. The data showed that the activity of TAC, SOD, CAT, and GSH were significantly reduced in the glutamate group (p<0.001), whereas TOS and MDA levels were significantly increased when compared to the control group. However, in the nicorandil groups, there was a marked enhancement in the activity of TAC, SOD, CAT, and GSH when compared to the glutamate group (p<0.001). Moreover, TOS and MDA levels were found to be substantially decreased in the nicorandil groups when compared to the glutamate group (p<0.001).

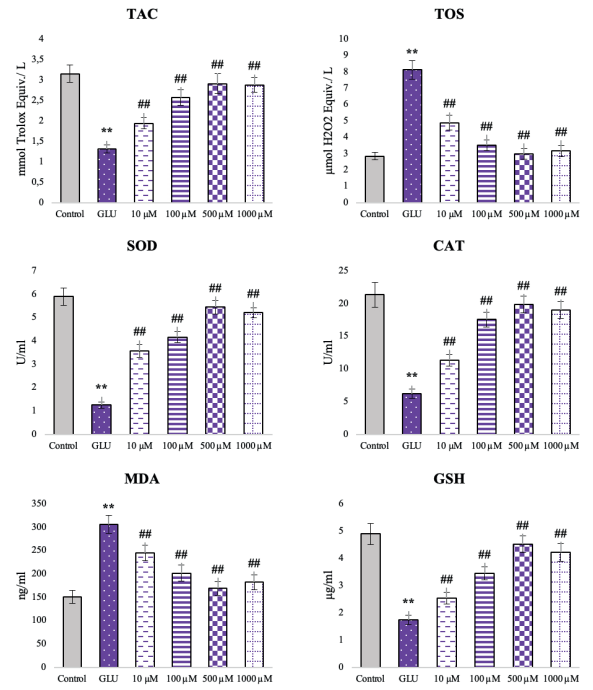


**Figure 2.** Effects of nicorandil on the oxidative stress parameters (TAC, TOS, SOD, CAT, MDA and GSH). Data are expressed as the means±SD. ** p<0.001 vs control group, ## p<0.001 vs GLU group

### Discussion

Nicorandil is a type of medication that functions as a nitric oxide donor and an ATP-sensitive potassium channel opener. This drug is utilized to enhance coronary blood flow in individuals who suffer from angina by exerting its effects. Many studies have shown its beneficial effects in cases of myocardial ischemia and diabetic myocardial damage, as well as in reducing the size of myocardial infarction [13]. K-ATP channels hyperpolarize the cell membranes of neurons, glia, and astrocytes and play a role in controlling glutamate concentrations and maintaining intercellular communication [14]. Recent studies have also shown that nicorandil can increase neurite outgrowth, neurogenesis, and the survival rate of neurons by preconditioning neuro stem cells [15]. It is also well documented in the literature that the Kir6.1 subunit of the K-ATP channel is effective against neuroinflammation [16].

The activation of the mitochondrial KATP channel by nicorandil can lead to an increase in potassium uptake in the mitochondrial matrix. This can reduce the accumulation of calcium ions and conserve the volume of the mitochondrial matrix. It is believed that nicorandil may offer protection to the cell from apoptosis induced by oxidative stress, as it reduces the production of ROS that can result from the increased volume of the mitochondrial matrix [7].

In a study on an Alzheimer's-like neuroblastoma cell culture model created by amyloid beta toxicity, it was observed that nicorandil protects cell viability by reducing Ca2+ uptake through its action on mitoKATP [17]. Based on these findings, it is believed that activation of mitochondrial KATP channels with nicorandil could be a useful tool in preventing cellular apoptosis and neuronal degeneration. Our observations parallel the results of this study, showing that nicorandil increases cell viability and protects against the toxic effects of glutamate.

Excitotoxicity resulting from glutamate is known to increase intracellular calcium influx, mitochondrial membrane permeability, and promote the formation of ROS [4]. It is expected that treatment with glutamate will result in an increase in MDA, LDH, and TOS levels, and a decrease in TAC, SOD, CAT, and GSH levels due to oxidative stress. Our data showed that treatment with nicorandil increased TAC, SOD, CAT, and GSH levels with increasing doses while decreasing MDA, LDH, and TOS levels.

In a cardiotoxicity study on nicorandil, it was stated that nicorandil may reduce the production of ROS by inhibiting the NADPH oxidase enzyme in addition to its effectiveness on mitochondrial KATP channels [18]. Also, some different researches have shown that nicorandil increases the levels of TAC, SOD, CAT, and GSH and decreases the levels of MDA, LDH, and TOS through its antioxidant activity [13,19-21]. We observed that Nicorandil reduced the amount of MDA, TOS, and LDH, while simultaneously increasing the levels of CAT, TAC, GSH, and SOD.

In another study Nagy et al. investigated the effects of diazoxide, another KATP channel opener, on neuronal preconditioning against excitotoxicity [22]. The study suggests that diazoxide-

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induced preconditioning involves a transient increase in superoxide anion production, which subsequently leads to reduced ROS availability upon glutamate exposure, thereby enhancing cell survival. This study and their study both emphasize the importance of KATP channel modulation in neuroprotection, but they differ in their proposed mechanisms. Although both of these drugs act as potassium channel openers, they may have different effects due to their distinct chemical structures. Numerous studies in recent years have demonstrated that drugs within the same group, routinely used today, can exhibit different therapeutic and side effects [23-27]. Therefore, it is evident that these two drugs, being part of the same group, may not always produce identical effects. These two studies provide valuable insights into the potential neuroprotective mechanisms of different drugs, nicorandil and diazoxide, in the context of glutamate-induced excitotoxicity. Further research and in vivo studies are necessary to validate these findings and determine the clinical implications of these drugs for treating neurotoxicity induced by glutamate.

## Conclusion

This study, which investigated the beneficial effects of nicorandil on glutamate toxicity, is a preliminary study for the treatment of many neurodegenerative diseases closely related to glutamate toxicity, and it is important to support the results with in vivo studies. The results indicate that nicorandil might have beneficial effects on the damage caused by glutamate toxicity in the brain. Based on these findings, nicorandil may be a useful treatment for preventing or reducing cell damage caused by glutamate toxicity and potentially could be used to treat neurotoxicity induced by glutamate.

**Conflict of Interests**
*The authors declare that there is no conflict of interest in the study.*

**Financial Disclosure**
*The authors declare that they have received no financial support for the study.*

**Ethical Approval**
*This investigation was performed with the endorsement of the Atatürk University Local Ethics Committee for Animal Experiments (75296309-050.01.04-2100303998).*

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