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Investigation of the effect of paracetamol against glutamate-induced cytotoxicity in C6 glia cells

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Abstract

Paracetamol is an active metabolite with analgesic and antipyretic properties of phenacetin, which is sold without a prescription in our country and in many countries. However, the effect of paracetamol on oxidative stress due to glutamate-induced cytotoxicity remains unclear. This study aims to investigate the effect of an appropriate dose of paracetamol on nitric oxide and increased oxidative stress as a result of glutamate-induced cytotoxicity in C6 cells. The cells were divided into 4 groups as Control group, Glutamate group, Paracetamol group, and Paracetamol+Glutamate group. Cell viability rate between groups was measured by XTT assay. Oxidative stress and antioxidant levels were measured with TOS and TAS elisa kits. Paracetamol at all concentrations significantly increased cell viability in C6 cells ($p < 0.001$). Paracetamol also increased TAS levels ($p < 0.01$) while significantly decreased TOS levels ($p < 0.001$). In addition, paracetamol was observed to decrease TNF-α and NO levels ($p < 0.001$). In conclusion, paracetamol has protective feature on glutamate-induced cytotoxicity in C6 glial cells by suppressing oxidative stress. The results of this study show that when the appropriate dose of paracetamol is used, it can be a crucial promoter agent in glutamate toxicity-induced neurodegeneration.

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1. Introduction

Glutamate, a proteinogenic amino acid, is a crucial neurotransmitter for excitatory signals in the mammalian central nervous system (CSN). However, the CSN also has many functional tasks with memory, cognition, and learning. Glutamate has an important role in the development of the CSN, containing migration, synapse induction, and differentiation [1]. However, intracellular glutamate concentration is in the millimolar (mM) interval, and excess glutamate is released out of the cell. Although glutamate is a necessary neurotransmitter for the continuation of the functions in the CNS, high concentrations of glutamate in the CNS cause neurodegeneration [2]. There can be many factors for neurodegeneration. One of them is excessive glutamate release. The result of excessive glutamate accumulating in the environment is the prolonged activation of glutamate receptors, as well as intracellular calcium overload, protease activation, mitochondrial dysfunction, increased ROS formation in response to an increase in intracellular calcium concentration, and neurodegeneration is observed in the CNS [3, 4]. In studies on glutamate toxicity, it has been associated with many neurodegenerative diseases and has been indicated to have a significant role in the damage mechanism [5, 6].

Glia cells are essential for providing a healthy environment for neurons in the CNS. In addition, excessive glia cell activation; it can cause many pathological conditions in the CNS such as ischemic stroke, Alzheimer's and Parkinson's Disease, Multiple Sclerosis. Oxidative stress (OS) is the most striking common feature in the formation of these damages [7]. Recent studies have shown that OS has significant actions on neuronal damage in various neurodegenerative diseases [8, 9]. It has been stated that the result of excessive extracellular glutamate level causes the depletion of the amount of intracellular glutathione (GSH), thus reducing the antioxidant level and causing the oxidant/antioxidant balance to deteriorate. It has been emphasized that this causes neuronal damage in the CNS through various mechanisms [10]. In a study conducted to understand the effect pathway of glutamate toxicity in C6 cells, which we used in our study, it was emphasized that the

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cells went to apoptosis due to the decrease in intracellular antioxidant level after glutamate incubation and the increase in OS [11]. Again, in a different in vitro study using C6 cells, apoptotic cell death was observed in these cells due to the increase in OS as a result of glutamate excitotoxicity [12]. Paracetamol is an active metabolite of phenacetin, which can be sold over-the-counter in our country and many other countries, with analgesic and antipyretic properties [13]. Paracetamol is a centrally acting drug that selectively inhibits nervous system cyclooxygenase (COX) [14], which inhibits prostaglandin synthesis. Other central mechanisms based on spinal serotonergic pathways may also be involved in the action mechanism of paracetamol. It is possible that the mechanism of action of paracetamol is using central mechanisms other than inhibition of prostaglandin synthesis. Animal studies have shown that the antinociceptive efficacy of paracetamol is reduced when serotoninergic pathways are damaged, depleted or blocked [15, 16]. Considering that increased glutamate-dependent cytotoxicity in the CNS causes neuronal damage, it will be important to develop a protective therapeutic approach against glutamate-induced excitotoxicity of neuronal cells. Paracetamol is one of the most widely used over-the-counter drugs in the world. These recently discovered effects have been significantly detected in vitro. It is thought that important therapeutic effects will be seen with the use of this drug in appropriate doses with new studies to be done [16, 17]. In this study, we investigated the effect of paracetamol against the increase in oxidative stress in the cytotoxicity model we created in glutamate-induced C6 cells. The main hypothesis of this study is that paracetamol reduces glutamate toxicity and oxidative stress on C6 cells. In this way, it will be shown that paracetamol has a protective feature on glutamate-induced cytotoxicity in C6 glial cells by suppressing oxidative stress.

2. Materials and Methods

2.1. Cell culture

Rat glioma cell (C6) line was used in the study because it is suitable for glutamate-dependent cytotoxicity [18]. C6 (CCL-107TM) cell line was obtained from the American Type Culture Collection (ATCC®, Manassas, VA, USA). The growth medium consisted of DMEM supplemented with FBS (10 %) and penicillin/streptomycin (1 %). C6 cells were cultured in 5 % CO_2 at 37 ºC. Paracetamol and glutamate were dissolved in isotonic saline and prepared freshly on the days of the experiment. Control group: no treatment was applied, Glutamate group: 10 mM glutamate was added and incubated for 24 hours, Paracetamol group: Different doses of paracetamol (5, 10, 20 and 40 µg/mL) were added and incubated for 24 hours, Paracetamol + Glutamate group: 1 hour after adding different doses of paracetamol (5, 10, 20 and 40 µg/mL) with 10 mM glutamate was incubated for 24 hours. In a study, glutamate toxicity group were formed to the single concentration of glutamate (10 mM) used to model the gliotoxicity [18]. Based on the literature, we used glutamate at a concentration (10 mM) in this study.

2.2. Cell viability assay

In order to investigate the protective effect of paracetamol against glutamate cytotoxicity on C6 cells, the cell viability rate was checked, for this purpose, XTT (Abcam, UK) assay was performed. C6 cells were seeded in 96-well plates (10×10^3 cells each well) in 100 µL DMEM. On the base of the litarature 1 hour after adding different doses of paracetamol (5, 10, 20 and 40 µg/mL), 10 mM glutamate was added to each well and incubated for 24 hours [15, 18]. After the 24 hour incubation period was over, 50 µL of the XTT reagent mixture was added to the C6 cells in a 96-well plate and then incubated at 37°C for 4 hours. The absorbance value for the XTT determination was measured at 450 nm using a microplate reader (Multiskan PLUS, Thermo Scientific). All tests were read by repeating three times.

2.3. Preparation of cells homogenates

All groups were transferred to different sterile falcon tubes. These tubes were centrifuged at 2000 rpm for 10 minutes. Tubes were brought into sterile laminar flow, supernatants were removed. The cell pellet remaining at the bottom of the tubes was diluted to $1x10^6$/mL by adding PBS (pH: 7.4). The cell structure was lysed via freeze-thaw repetition and the cytoplasmic ingredients were withdrawn. The mixture was centrifuged at 4000 rpm for 10 minutes. The supernatant remaining in the upper part of the tubes was taken with the help of sterile pipettes and collected in various sterile tubes. Total protein levels in the study groups were measured with the Bradford protein assay kit (Merck Millipore).

2.4. Measurement of total antioxidant status (TAS), total oxidant status (TOS), tumor necrosis factor-alpha (TNF-α) and nitric oxide (NO) levels in the C6 cells

Glutamate toxicity induction of TAS, TOS, TNF-α and NO levels in the supernatants of C6 cells was determined by ELISA kits (BT Lab). These analyzes were performed considering the protocols determined by the companies for commercial kits. Samples

incubated at 37 °C for 60 minutes were placed in 96-well plates. Standard and supernatant samples were inserted to the plate and incubated for 60 minutes. After the washing step, staining solutions were added and incubated for 15 minutes. Stop solution was inserted and absorbances were read at 450 nm on an ELISA microplate reader (Thermo Fisher Scientifc).

2.5. Statistical analysis

Study results were indicated as mean ± SEM (standard error of mean). Data analyzes were performed with SPSS Version 22. Data were evaluated using a one-way analysis of variance (ANOVA) and a posthoc Tukey test. $p < 0.05$ was determined statistically significant.

3. Results

3.1. Effect of paracetamol on C6 cell viability

In this study, cell viability was investigated in study groups using different doses to determine the effect of paracetamol against glutamate-toxicity. For this, XTT cell viability kit was used. Cell viability was determined in C6 cells treated with both control and glutamate at different doses (5-40 µg/mL) for paracetamol. C6 cells were initially pre-treated with doses of paracetamol (5, 10, 20 and 40 µg/mL) for 60 minutes and incubated with or without glutamate (10 mM) for the next 24 hours. Pre-incubation of C6 cells with glutamate for 24 hours substantially reduced cell viability compared to untreated cells (control group) ($p<0.001$; Figure 1). However, paracetamol doses tested appeared to increase cell survival in C6 cells compared to C6 cells incubated with glutamate ($p<0.001$; Figure 1)

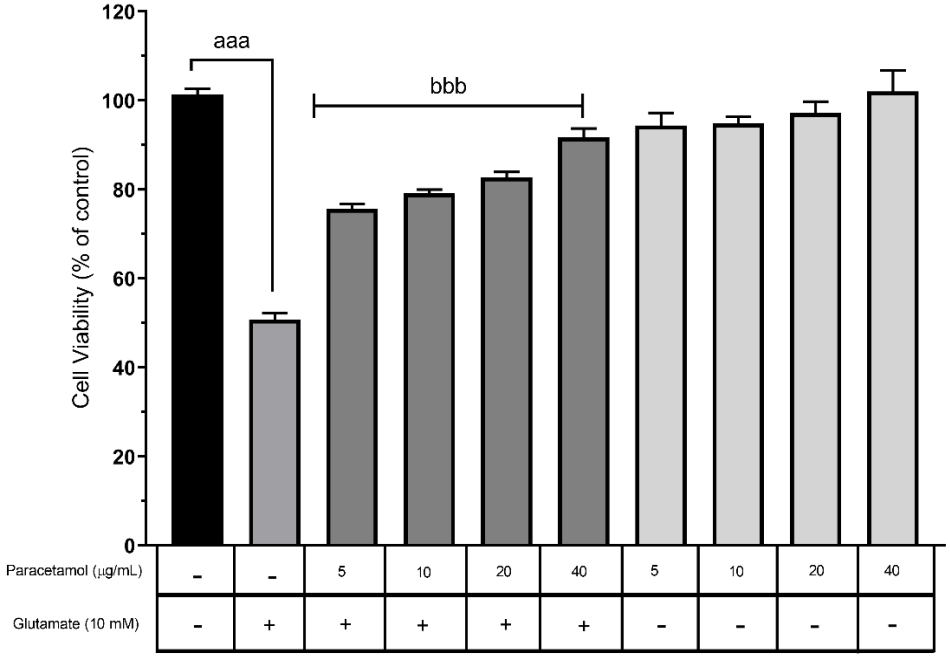

Figure 1. Effect of paracetamol on cell viability after glutamate-induced cytotoxicity in the C6 cells. $^{aaa}p \leq 0.001$ as compared to control-untreated group; $^{bbb}p \leq 0.001$ compared to glutamate-treated group.

3.2. Effect of paracetamol on TAS and TOS levels in C6 cells

The changes in TAS and TOS levels in cells against glutamate cytotoxicity of paracetamol in the groups formed were measured with the Elisa kits. TAS levels were significantly reduced between the groups when the glutamate-treated group was compared to the control and Paracetamol+Glutamate groups ($p<0.001$; Figure 2A). There was no statistically significant difference between the control group and the Paracetamol+Glutamate group (Figure 2A). When the glutamate-treated group was compared to the control and Paracetamol+Glutamate groups between the groups, it was observed that the TOS level increased considerably ($p<0.001$; Figure 2B). No statistically significant difference was found between the control group and the Paracetamol+Glutamate group (Figure 2B).

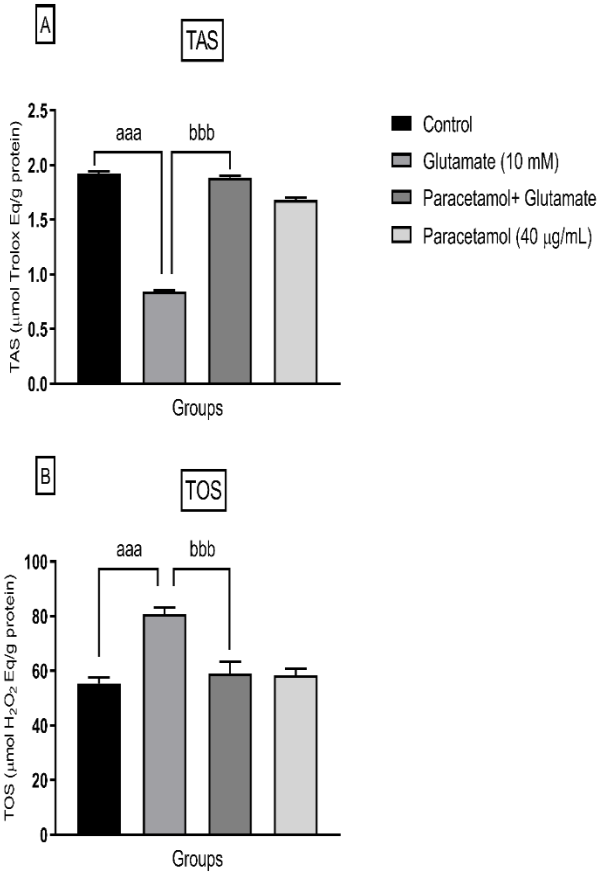

Figure 2. Effect of paracetamol on TAS and TOS levels in C6 cells after glutamate-induced cytotoxicity. $^{aaa}p \leq 0.001$ as compared to control group; $^{bbb}p \leq 0.001$ compared to glutamate-treated group.

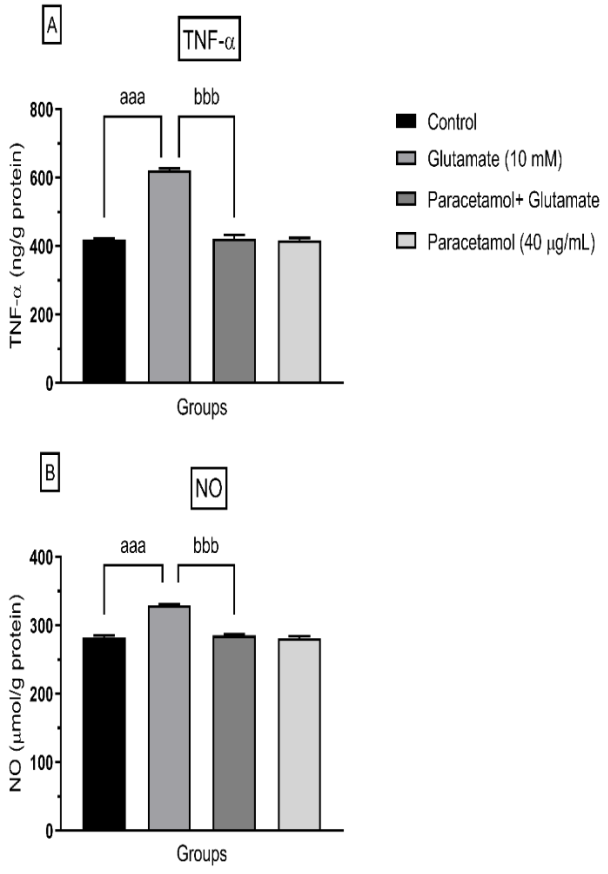

Figure 3. Effect of paracetamol on TNF-α and NO levels in C6 cells after glutamate-induced cytotoxicity. $^{aaa}p \leq 0.001$ as compared to control group; $^{bbb}p \leq 0.001$ compared to glutamate-treated group.

3.3. Effect of paracetamol on TNF-α and NO levels in C6 cells after glutamate-induced cytotoxicity

ELISA commercial kits were used to investigate the effect of paracetamol on TNF-α and NO levels in glutamate-induced C6 cells. TNF-α levels were substantially increased between the groups when the glutamate-treated group was compared to the control and Paracetamol+Glutamate groups ($p<0.001$; Figure 3A). There was no statistically considerable difference between the control group and the Paracetamol+Glutamate group ($p>0.05$; Figure 3A). When the glutamate-treated group was compared to the control and Paracetamol+Glutamate groups between the groups, the NO level increased significantly ($p<0.001$; Figure 3B). No statistically significant difference was found between the control group and the Paracetamol+Glutamate group ($p>0.05$; Figure 3B).

4. Discussion

In our study, the effect of pre-treatment of paracetamol using different doses against glutamate-dependent toxicity, which we induced in vitro, was investigated. It was observed that pretreatment with paracetamol increased cell viability and reduced cell death against glutamate-dependent cytotoxicity in C6 cells (Figure 1). Also, Paracetamol pretreatment decreased TOS levels and increased TAS level in C6 cells. In addition, paracetamol pretreatment was also observed to suppress TNF-α and NO levels. Although glutamate is one of the most crucial neurotransmitters for CSN, its depolarization in neurons causes excitotoxicity if its extracellular concentration increases. It has been mentioned in the literature that glutamate in excitotoxicity causes NOS regulation, mitochondria dysfunction, ROS production, ER stress as a result of intracellular Ca^{2+} increase [18, 19]. In the study conducted by Gundogdu et al, a significant decrease in cell viability was detected in primary cortical neuron cells with glutamate toxicity. An increase in cell

viability was detected following the application of parietin to primary cortical neuron cells with toxicity [1]. In a study performed by Das et al melatonin receptor 1 appeared to be involved in the protection of C6 cells from excitotoxic and oxidative damage [12]. Similarily in our study, paracetamol had significant protective effect against glutamate-induced cytotoxicity and oxidative stress. In a study the effect of salmon calcitonin against glutamate-induced cytotoxicity was investigated in C6 cell line. It was observed that there was an increase in NOS and NO levels in cells [18]. In another study examining the protective effect of Thiamine against glutamate-induced cytotoxicity in C6 cell line, an increase was observed in MDA levels after glutamate incubation, while a decrease was observed in SOD and CAT levels. It was observed that the use of thiamine increased cell viability by reducing the oxidant damage caused by the use of glutamate [20]. In a study performed by Park et al, it was determined that glioma cells with glutamate toxicity increased OS, mitochondrial dysfunction and ER stress. It was observed that after the use of alpha-lipoic acid, OS, mitochondrial dysfunction and ER stress were reduced [21]. In support of the studies in the literature, we also observed that the TAS level increased and TOS level decreased in the paracetamol group compared to the glutamate group in C6 cells, in the use of paracetamol against cytotoxicity caused by glutamate in our study (Figure 2).

TNF-α is a very important proinflammatory cytokine in the regulation of inflammation in the CNS. It is actually expressed at low levels by different other cells, including neurons, without a pathological condition. However, in case of any pathological or inflammation, its secretion also increases in other cells, especially in microglia cells, which are local defense cells in the CNS. For this reason, it is accepted as an important indicator in determining a damage mechanism that may occur in the CNS [9, 23]. Marchetti et al, found very important findings about TNF- α expression in their study on glutamate toxicity. As a result, they stated that it may be an inflammation marker [23]. In our study, we observed that the TNF- α level, which increased after glutamate toxicity, decreased after paracetamol treatment. This shows that paracetamol can be an important therapeutic agent for glutamate toxicity (Figure 3A). In a study performed by Lesage et al on primary hippocampal cell culture, they showed that there is an increase in NO with glutamate toxicity. They argued that preventing the increased NO level could be an important approach in reducing the damage [24]. In our study, we observed that the NO level, which increased after glutamate toxicity, decreased after paracetamol treatment (Figure 3B).

Our study results and literature studies showed that paracetamol can reduce cell death in C6 cells. This protective effect of paracetamol appears to occur with the suppression of oxidative stress and NO pathways. Therefore, the use of appropriate dose of paracetamol may be protective in CNS disorder and may be selected as a beneficial therapeutic agent in neurodegenerative disorders.

5. Conclusions

The findings of this study showed that the use of appropriate dose of paracetamol reduced cellular damage against glutamate-dependent cytotoxicity in C6 cells. It is seen that this protective effect of paracetamol is revealed by the suppression of oxidative stress and NO pathways. Therefore, the use of appropriate doses of paracetamol may be protective against glutamate toxicity that may occur in CNS disorders and can be used as a useful therapeutic agent in neuronal damage-related damage. However, further in vitro and in vivo studies are needed to answer questions about the possible mechanisms of action of paracetamol in glutamate toxicity.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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