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Investigation of the effect of sugammadex on glutamate-induced neurotoxicity in C6 cell line and the roles played by nitric oxide and oxidative stress pathways

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Abstract

This experiment was intended to evaluate the effect of sugammadex on the cytotoxicity induced by glutamate, involving the nitric oxide and oxidative stress pathways. C6 glioma cells were used in the study. Glutamate was given to cells in the glutamate group for 24 h. Sugammadex at different concentrations was given to cells in the sugammadex group for 24 h. Cells in the sugammadex + glutamate group were pre-treated with sugammadex at various concentrations for 1 h and then exposed to glutamate for 24 h. XTT assay was used to assess cell viability. Levels of nitric oxide (NO), neuronal nitric oxide synthase (nNOS), total antioxidant (TAS), and total oxidant (TOS) in the cells were calculated using commercial kits. Apoptosis was detected by TUNEL assay. Sugammadex at concentrations of 50 and 100 μg/mL significantly enhanced the cell viability in C6 cells after the cytotoxicity induced by glutamate ($p < 0.001$). Moreover, sugammadex considerably decreased the levels of nNOS NO and TOS and the number of apoptotic cells and increased the level of TAS ($p < 0.001$). Sugammadex has protective and antioxidant properties on cytotoxicity and could be an effective supplement for neurodegenerative diseases such as Alzheimer and Parkinson if further research in vivo supports this claim.

KEYWORDS

C6 rat glioma, glutamate, nitric oxide, oxidative stress, sugammadex

1 | INTRODUCTION

Glutamate, the primary excitatory neurotransmitter in the central nervous system, is implicated in neurodevelopment as well as activities such as synaptic transmission regulation, sensory activity, memory, learning, and motor function [1]. In a number of neurodegenerative disorders such as Parkinson's disease, Huntington's disease, dementia complex, amyotrophic lateral sclerosis, and Alzheimer's disease, overstimulation of both ionotropic and metabotropic glutamate receptors has been related to neuronal damage [2]. Neurological trauma, hypoglycemia, stroke, and epilepsy induce excitatory dysregulation and, thus, massive brain cell death [3]. Excitotoxicity, which is generated by excessive glutamate stimulation and leads to excessive calcium ion influx into the cell, is the main mechanism in the development of many disorders in the nervous center system [4]. Excessive calcium entry into cells causes mitochondrial dysfunction, which raises intracellular nitric oxide levels and promotes cell apoptosis [5]. In vitro, excitotoxicity and cystine transport inhibition-related oxidative stresses have been considered two important mechanisms of glutamate neurotoxicity [6]. If left untreated, excessive glutamate exposure results in membrane depolarization, which can lead to

Abbreviations: FBS, fetal bovine serum; GMP, guanosine monophosphate; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; TAS, total antioxidant status; TOS, total oxidant status.

cell death. Glutamate causes oxidative stress by blocking cystine transport and increasing reactive oxygen species production at the same time. The formation of oxidative stress by glutamate has been demonstrated to be the most common cytotoxic route in C6 cell lines. The disruption of intracellular redox homeostasis caused by glutamate appears to be a substantial factor in cellular damage in vivo [6].

Cyclodextrins (CDs) are cyclic oligosaccharides with a lipophilic central cavity and a hydrophilic outer surface. CDs are made up of glucopyranose units that are organized in a torus-like pattern, with secondary hydroxyl groups on one side and all primary hydroxyl groups on the other. The torus shells of α, β, and γ-CD contain 6, 7, and 8 cyclic glucopyranose units, respectively. The primary therapeutic application of CDs in the pharmaceutical industry is to increase the solubility, bioavailability, and physical stability of active ingredients by generating inclusion complexes [7]. Cyclodextrins have previously been proven to have neuroprotective properties [8].

Sugammadex is a modified gamma cyclodextrin compound that has just received FDA approval for neuromuscular pharmacology. Neuromuscular inhibiting drugs can be encapsulated and removed from the muscle-nerve junction by sugammadex. It is used to reverse neuromuscular inhibition selectively and quickly [7]. Sugammadex's chemical structure and high molecular weight make it difficult to pass through the blood–brain barrier and the placenta. Even though this property precludes sugammadex derivatives from passing through the blood–brain barrier at significant concentrations, this situation may be changed in patients with neurodegenerative diseases such as Alzheimer's disease, ischemia, Parkinson's disease and inflammation, or an immature nervous system [9]. This drug has been found to have neuroprotective characteristics in the brain ischemia perfusion model in the literature [10]. However, the effect of sugammadex on glutamate-induced cytotoxicity and the mechanisms behind it remain unknown. In this work, the effect of sugammadex on glutamate-induced cytotoxicity in C6 glial cells and potential mechanisms involved in this effect such as oxidative stress and nitric oxide pathways were examined.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Because it has suitable glutamate-induced cytotoxicity, the C6 Glioma (CRL107) cell line was employed in this research [11]. This cell line was purchased from the American Type Culture Collection. The cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin. DMEM was obtained from Thermo Fisher Scientific, Altrincham, UK. FBS, L-glutamine, and penicillin/streptomycin were obtained from Sigma-Aldrich Co., St Louis, MO, USA. The cells were kept at 37°C in a humidified environment containing 5% CO_2.

2.2 | Drug administration

Before treatment, sugammadex and glutamate were dissolved in DMEM, and stock solutions were prepared. Sugammadex and glutamate were obtained from Sigma-Aldrich Co., St Louis, MO, USA.

2.3 | Glutamate-induced cytotoxicity

To study the effect of sugammadex on glutamate-induced cytotoxicity, four cell groups were created. There was no treatment for the control group. The glutamate group's cells were given 10 mM glutamate for 24 h. According to the literature, the glutamate concentration (10 mM) that causes the death of 50% of glial cells was chosen [12]. Sugammadex was given to cells in the sugammadex group at various concentrations (12.5, 25, 50, and 100 μg/mL) for 24 h. A pilot experiment was carried out before this study was conducted. Accordingly, sugammadex concentrations were selected. Sugammadex + glutamate group's cells were pre-treated with various doses of sugammadex (12.5, 25, 50, and 100 μg/mL) for 1 h before being exposed to 10 mM glutamate for 24 h.

2.4 | Cell viability assay

The XTT assay was used to determine cell viability (Roche Diagnostic, MA, USA). In 100 μL DMEM, C6 Glioma cells were planted at a density of 1×10^4 cells per well in 96-well plates and cultured overnight before being treated with sugammadex. The glutamate-induced cytotoxicity procedure was carried out as described previously [13]. The medium was withdrawn after 24 h of incubation, and the wells were rinsed twice with phosphate-buffered saline (PBS). In the final phase, 100 μL of DMEM without phenol red and 50 μL mixture of XTT labeling solution were added to each well, and the plates were kept at 37°C for 4 h. After shaking the plates, the absorbance was measured at 450 nm using an ELISA microplate reader (Thermo Fisher Scientific, Altrincham, UK). All of the tests were repeated three times, and cell viability was calculated as a percentage of viable cells relative to the control [14].

2.5 | TUNEL assay

The TUNEL assay inserts biotinylated nucleotides to the strand breaks observed in the DNA of apoptotic

cells using an enzyme (terminal deoxynucleotidyl transferase). The TUNEL assay was carried out according to the manufacturer's instructions. Briefly, glutamate-treated, sugammadex-treated, sugammadex + glutamate-treated, or nontreated C6 cells were cultured in 6-well plates for 24 h. The culture media was aspirated after the overnight incubation, and the cell layers were trypsinized. The trypsinized cells were reattached to 0.01% polylysine-coated slides, fixed with 4% methanol-free formaldehyde solution, and stained using the TUNEL system methodology (BioVision, Milpitas, CA, USA). A fluorescent microscope with an excitation wavelength of 450–500 nm and a detection wavelength of 515–565 nm was used to examine the stained cells. In each experiment, 100 cells were counted to assess the percentage of cells that were undergoing apoptosis.

2.6 | Preparation of cell homogenates

Each group's cells were collected in sterile tubes. The supernatants were removed after centrifugation at 2000 rpm for about 10 min. The cells in the tubes were suspended by diluting the cell suspension with PBS (pH: 7.4) to a cell density of approximately 1 million/ml. To let out the interior components, the cells were destroyed by repeated freeze–thaw cycles. To avoid changing in protein activity, the freeze–thaw cycles were repeated twice [13]. At a temperature of 4°C, they were centrifuged for 10 min at 4000 rpm. The supernatants were then collected and subjected to biochemical analysis. Total protein levels in the samples were determined using the Bradford protein assay kit (Merck Millipore, Darmstadt, Germany).

2.7 | Measurement of neuronal nitric oxide synthase (nNOS) and nitric oxide (NO) levels in the cells

The levels of nNOS and NO in the supernatants of cells for each group were measured using ELISA commercial kits (BT Lab, Shanghai, China). The operation procedures followed the manufacturer's guidelines. In a nutshell, the standard and cell samples were placed in the plate and incubated at 37°C for 60 min. The staining solutions were applied after the washing process and kept for 15 min at 37°C. The absorbance was measured at 450 nm using an ELISA microplate reader after the stop solution was added (Thermo Fisher Scientific, Altrincham, UK). To determine the value of samples, standard curves were plotted. Within and between plates, the coefficients of variation were less than 10%.

2.8 | Evaluation of TAS and TOS levels

TAS and TOS levels in cell supernatants were determined using an automated test method designed by Erel [15]. The total antioxidant status assay relies on determining the absorbance of colored dianisidyl radicals during free radical production to control the reaction fraction of free radicals. Antioxidants in the samples should prevent the samples from coloring in proportion to their quantities. The results were calculated as μmol Trolox Eq/mg protein. In the total oxidant status test, on the other hand, when the medium contains adequate oxidizers, ferrous ions are oxidized to ferric ions, allowing TOS levels to be evaluated by measuring the level of ferric ions using orange xylenol. Hydrogen peroxide was used to standardize the test. The values were calculated as μmol H_2O_2 Eq/mg protein. Total protein levels in samples were determined using a Bradford protein assay kit (Merck Millipore, Darmstadt, Germany).

2.9 | Statistical analysis

The results were expressed as a mean ± standard error of the mean. The data analyses were performed with SPSS version 25.0 for Windows. The statistical significance of the differences was evaluated by one-way analysis of variance (ANOVA) followed by post hoc Tukey multiple comparison test. $P < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Effect of sugammadex on cytotoxicity induced by glutamate in C6 cell line

The XTT assay was used to assess the protective properties of sugammadex against the cytotoxicity induced by glutamate in C6 cells. In this investigation, sugammadex at different concentrations (12.5–100 μg/mL) was applied to test the effect of this drug on cell viability in both control and glutamate-treated C6 cells. The cells were first treated with various concentrations of sugammadex (12.5–100 μg/mL) for 1 h before being incubated with or without 10 mM glutamate for another 24 h. Pre-incubating C6 cells with glutamate for 24 h reduced cell survival (57.33 ± 1.47%) substantially when compared to untreated control cells ($P < 0.001$; Figure 1). Sugammadex at concentrations of 50 and 100 μg/ml, on the other hand, improved cell viability (85.66 ± 2.14% and 83 ± 1.59% subsequently) in C6 cells as compared to glutamate-treated cells ($P < 0.001$; Figure 1). Moreover, as compared to untreated control cells, sugammadex alone had no

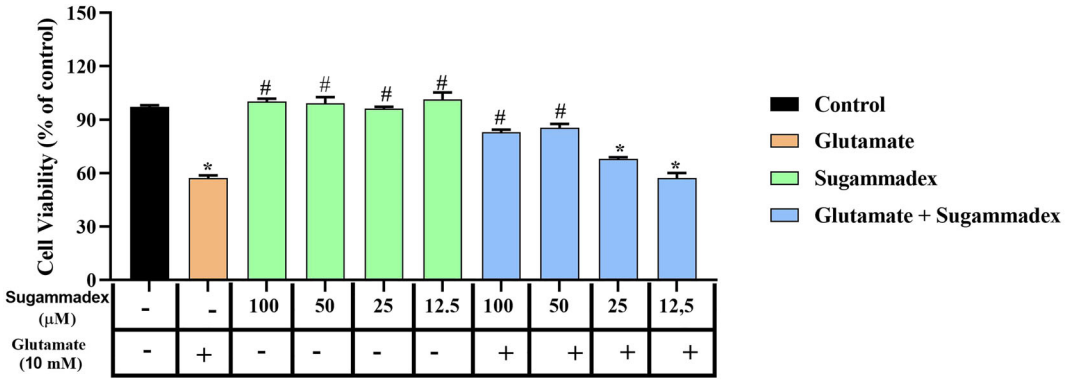

FIGURE 1 Effect of sugammadex at different concentrations on cell survival after glutamate-induced cytotoxicity in C6 cells. The data are expressed as mean ± SEM. *p < 0.001 as compared to the untreated control group; #p < 0.001 as compared to glutamate-treated group.

effect on C6 survival at any concentration (P > 0.05; Figure 1).

3.2 | Effect of sugammadex on apoptosis after cytotoxicity induced by glutamate in C6 cells

The TUNEL assay was used to assess the anti-apoptotic properties of sugammadex after cytotoxicity induced by glutamate in C6 cells. With TUNEL/DAPI double labeling, apoptotic cells display green fluorescent labeling with TUNEL labeling, and live cells display blue fluorescence with DAPI labeling. In comparison to untreated control cells, pre-incubating C6 cells with glutamate for 24 h significantly increased the number of apoptotic cells (60 ± 1.47%) (P < 0.001; Figure 2). Sugammadex at a concentration of 50 μg/mL, on the other hand, reduced the number of apoptotic cells (21 ± 1.32%) in C6 cells as compared to glutamate-treated cells (P < 0.001; Figure 2). However, sugammadex at concentration of 50 μg/mL alone did not cause a significant anti-apoptotic effect in the C6 cells as compared to the control (P > 0.05; Figure 2).

3.3 | Effect of sugammadex on nNOS and NO levels after cytotoxicity induced by glutamate in C6 cells

The ELISA tests were used to see how sugammadex affected nNOS and NO after cytotoxicity induced by glutamate in C6 cells. The cells were treated with 50 μg/mL sugammadex for 1 h before being incubated or not with 10 mM glutamate for another 24 h. In comparison to untreated control cells, pre-incubating C6 cells with glutamate for 24 h substantially increased the levels of nNOS (156.47 ± 1.71 ng/g protein) and NO (1034.69 ± 8.28 μM/g protein) (P < 0.001; Figure 3). Sugammadex at a concentration of 50 μg/mL, on the other hand, dramatically lowered the levels of nNOS (126.48 ± 1.94 ng/g protein) and NO (923.20 ± 3.51 μM/g protein) in C6 cells as compared to the glutamate-treated cells (P < 0.001; Figure 3). However, sugammadex at a concentration of 50 μg/mL alone did not cause a significant alteration in the levels of nNOS and NO in the C6 cells as compared to the control (P > 0.05; Figure 3).

3.4 | Effect of sugammadex on TAS and TOS levels after cytotoxicity induced by glutamate in C6 cells

The cells were treated with 50 μg/mL sugammadex for 1 h before being incubated or not with 10 mM glutamate for another 24 h. In comparison to untreated control cells, pre-incubating C6 cells with glutamate for 24 h substantially decreased TAS levels (0.84 ± 0.01 μmol Trolox Eq/mg protein) (P < 0.001; Figure 4a). Sugammadex at a concentration of 50 μg/mL, on the other hand, dramatically elevated TAS levels (1.73 ± 0.02 μmol Trolox Eq/mg protein) in C6 cells as compared to the glutamate-treated cells (P < 0.001; Figure 4a). Moreover, pre-incubating the C6 cells with glutamate for 24 h substantially elevated TOS levels (79.44 ± 0.82 μmol H_2O_2 Eq/mg protein) as compared to the control (P < 0.001; Figure 4b). Sugammadex at a concentration of 50 μg/mL, on the other hand, dramatically lowered TOS levels (54.67 ± 1.31 μmol H_2O_2 Eq/mg protein) in C6 cells as compared to the glutamate-treated cells (P < 0.001; Figure 4b). However, sugammadex at a concentration of 50 μg/mL alone did not cause a significant alteration in the levels of TAS and TOS in the C6 cells as compared to the control (P > 0.05; Figure 4a,b).

4 | DISCUSSION

The effects of sugammadex on glutamate-induced cytotoxicity in C6 cells were investigated for the first

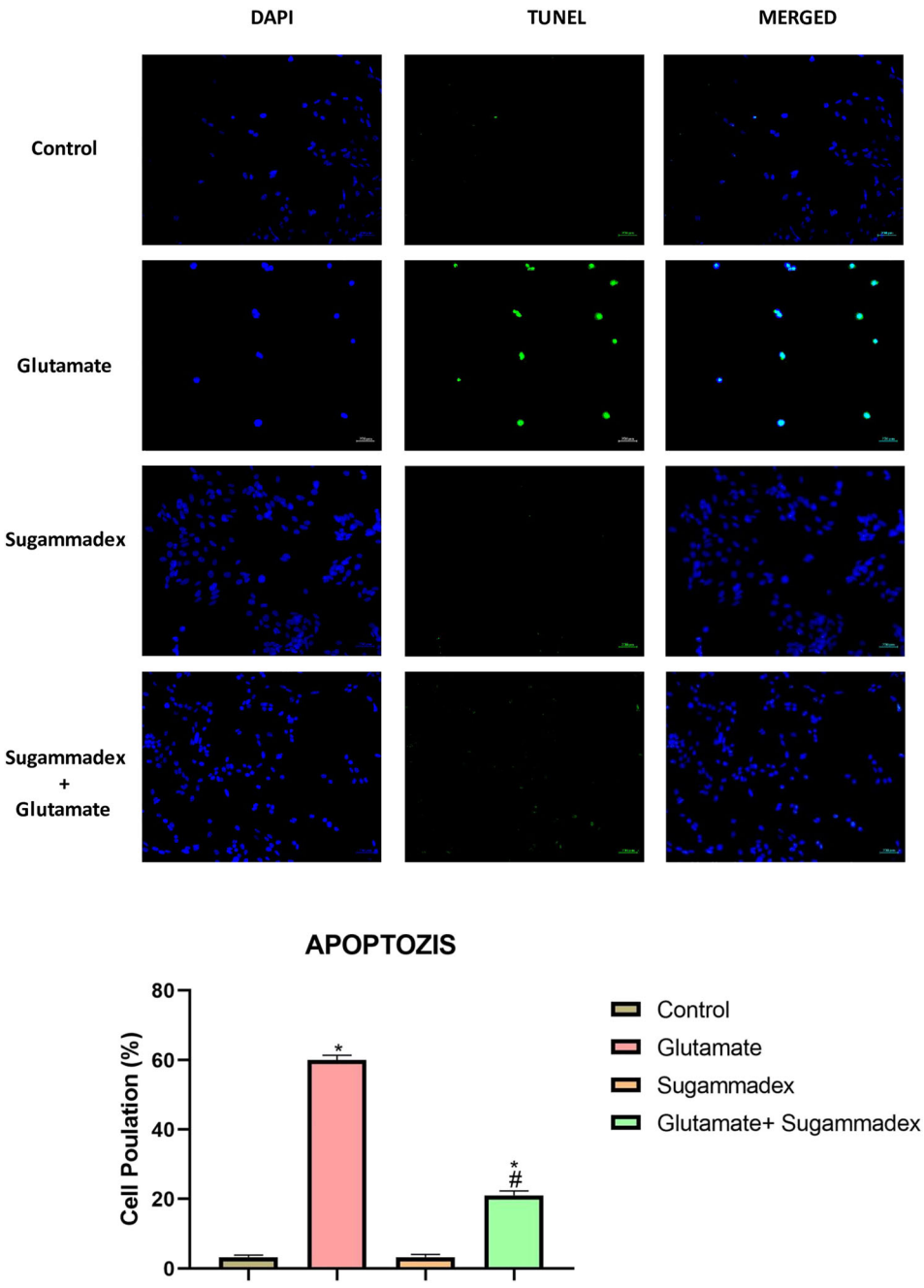

FIGURE 2 Effect of 50 μM sugammadex on apoptosis after glutamate-induced cytotoxicity in C6 cells. Cell apoptosis was determined with a TUNEL assay. Apoptotic cells display green fluorescence with a TUNEL labeling, and live cells display blue fluorescence with DAPI labeling. One hundred cells were counted to assess the percentage of cells that were undergoing apoptosis. The data are expressed as mean ± SEM. *$p < 0.001$ as compared to untreated control group; #$p < 0.001$ as compared to the glutamate-treated group.

time in this study. Sugammadex administration improved cell survival and decreased cell death in C6 cells after glutamate-induced cytotoxicity. Furthermore, sugammadex decreased the nNOS, NO, and TOS levels and increased TAS levels after glutamate-induced cytotoxicity in the C6 cells.

The study conducted by Mucuoglu et al. revealed that sugammadex, an agent that reverses the impacts of neuromuscular blocking drugs, has neuroprotective properties and is as efficient as mannitol [16]. Sugammadex produced neuroprotective benefits and reduced the number of apoptotic neurons in a rat model of cerebral ischemia-reperfusion injury [10]. Sugammadex is a synthetic γ-cyclodextrin and the first member of the SRBA (selective relaxant binding agents) class of neuromuscular reversal medicines. Cyclodextrins are cyclic dextrose units that are linked together by 1–4 glycosyl bonds and are made from starch or starch derivates utilizing cyclodextrin glycosyltransferase [17]. Cortical neurons are protected from glutamate excitotoxicity and oxygen–glucose deprivation by a variety of cyclodextrin groups [18]. It has been discovered that methyl-β-cyclodextrin protected the hippocampus areas from the effects of anoxia [19]. Abulrob et al. investigated the neuroprotective properties of the cyclodextrin 2-hydroxypropyl-

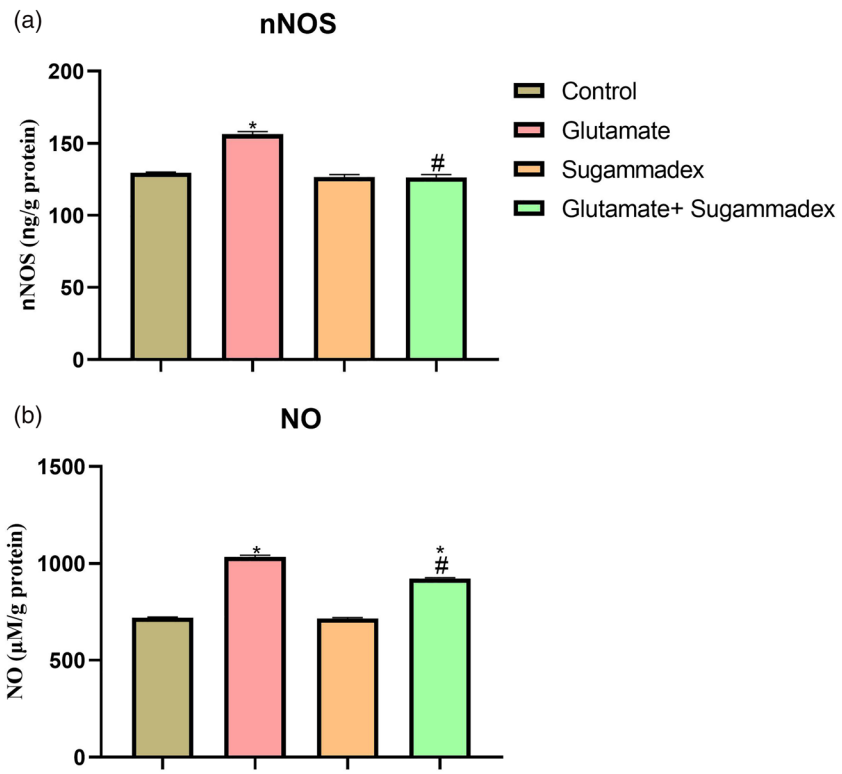

FIGURE 3 Effect of 50 µM sugammadex on neuronal nitric oxide synthase (nNOS) and nitric oxide (NO) levels after glutamate-induced cytotoxicity in C6 cells. The data are expressed as mean ± SEM. *$p < 0.001$ as compared to the untreated control group; #$p < 0.001$ as compared to glutamate-treated group.

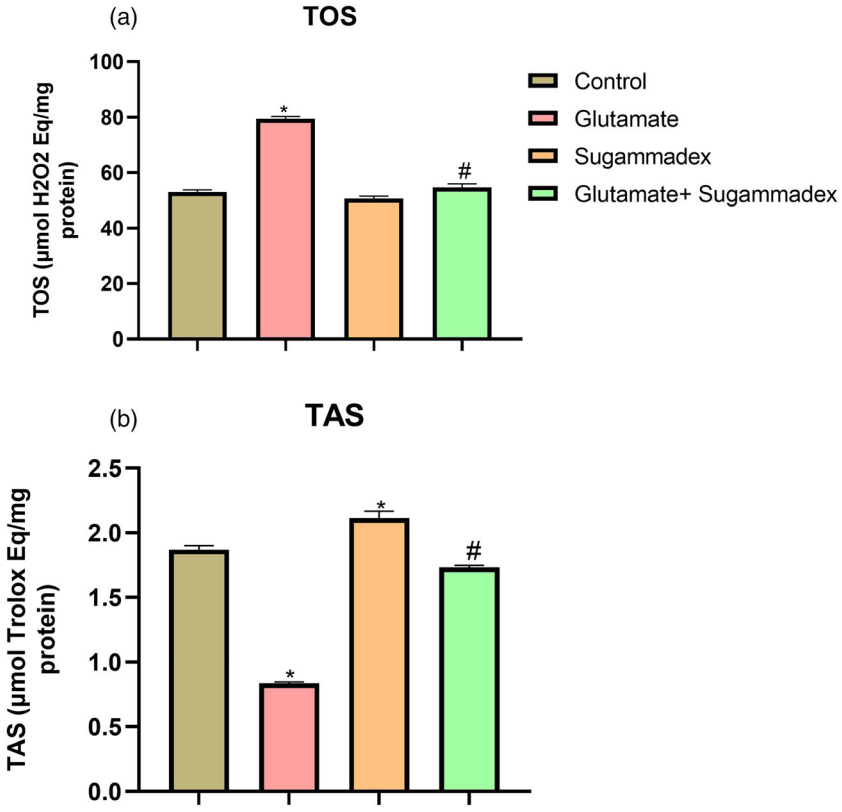

FIGURE 4 Effect of 50 µM sugammadex on total antioxidant (TAS) and total oxidant (TOS) levels after glutamate-induced cytotoxicity in C6 cells. The data are expressed as mean ± SEM. *$p < 0.001$ as compared to the untreated control group; #$p < 0.001$ as compared to glutamate-treated group.

β-cyclodextrin (HP-CD) [7]. Yao et al. performed a study in rats in which they were given HP-CD and discovered that cyclodextrins showed significant neuroprotective benefits [20]. According to Frank et al., methyl-β-cyclodextrin reduces neuronal excitability in the hippocampal area because it can break away cholesterol in the postsynaptic region, disrupting glutamate receptors [8]. In our study, sugammadex increased cell survival and reduced apoptosis in C6 cells after glutamate-induced cytotoxicity.

NO is an important neuromodulator in the central nervous system. Endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS) are the three types of nitric oxide synthases that synthesize NO from the oxidation of the L-arginine amino acid [21].

The nNOS is widely distributed in neurons [22], and it was found that C6 cells express it [23]. The induction of nNOS causes a rise in NO in the neurons, which causes the soluble guanylate cyclase (sGC)/guanosine monophosphate (GMP) pathway to be activated. The cGMP is a secondary messenger that triggers glutamate release and excitement in the neurons [24]. In addition, nNOS/NO has a role in glutamate-induced cytotoxicity [11, 13]. The elevation in nNOS and NO levels in the cells after glutamate-induced cytotoxicity was uncovered in this work. On the other hand, sugammadex reduced elevated levels of nNOS and NO after glutamate-induced cytotoxicity in C6 cells.

An imbalance between oxidants and antioxidant defense mechanisms is known as oxidative stress. This imbalance results in excessive ROS generation, which damages tissues and disrupts the organism's physiological function [25]. Furthermore, studies suggest that oxidative stress is a key factor in the development of neurodegenerative illnesses and CNS disorders [26,27].

The elevation in TOS levels and reduction in TAS levels in the cells after glutamate-induced cytotoxicity was uncovered in this work. Despite this, sugammadex displayed antioxidant properties elevating TAS levels and also reduced oxidative damage decreasing TOS levels after glutamate-induced oxidative damage in the C6 cells.

Several authors have recently recommended the use of cyclodextrins as "primary antioxidants" for controlling enzymatic browning in various fruits. Because of the protective effect given by cyclodextrins against ascorbic acid oxidation, cyclodextrins can also improve ascorbic acid's capacity to resist enzymatic browning. İn addition, cyclodextrins can protect the carotenoids from reactive oxygen species [28]. According to the study conducted by Abedelnasser et al., cholesterol-extracting cyclodextrins protect neurons by altering the distribution and signaling of the NMDA NR2B–PSD-95–nNOS complex [7]. Based on these findings, it is possible that other cyclodextrins forms, such as sugammadex, have protective benefits against glutamate-induced cytotoxicity.

The study has a potential limitation. Although the results obtained from this study were interesting and important, there is still a need for a deeper investigation of the molecular mechanisms that provide more accurate explanations for these results

5 | CONCLUSION

Sugammadex has a protective impact on glial cell survival after glutamate-induced cytotoxicity in C6 cells, according to our data. Because glial cells are important in neurodegenerative diseases, sugammadex could be used as a complementary therapy in the treatment of neurodegenerative diseases. However, further research is needed to support this claim.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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