Introduction

Glutamate, considered the most important stimulating neurotransmitter of the central nervous system, has a very critical role in the regulation of many cognitive functions, including synaptic plasticity, learning, and memory. Glutamate is found in the central nervous system (CNS) in millimolar concentrations. The synaptic activity gives rise to an enhancement in glutamate concentration in the synaptic cleft, but extracellular glutamate concentration is preserved with the uptake of glutamate by glutamate transporters. Although glutamate has essential for brain functions, its high concentration in the CNS causes the neurotoxic effect of glutamate. As a result of excessive glutamate release, calcium overload caused by long-term activation of glutamate receptors leads to excitotoxicity and this situation has an important role in neurodegeneration. Protease activation, mitochondrial dysfunction, and an increase in reactive oxygen species occur in response to the increase in intracellular calcium concentration, and this triggers neuronal cell death.

Astroglial cells are non-neuronal cells that maintain homeostasis as well as support and protect neurons in the CNS. Moreover, activation of the glial cells causes neuron inflammation and oxidative stress that destroy the neuronal cells. Therefore, the health of glial cells plays an important role in the development of neurodegenerative disorders, especially Alzheimer’s disease and Parkinson’s disease. In particular, excessive extracellular glutamate impairs not only neurons but also astroglial cells. Glial and neuronal cell death, which is the underlying cause of progressive neuropathological processes, is induced by intracellular increased oxidative stress and endoplasmic reticulum (ER) stress. The rise in the glutamate concentration causes oxidative stress and also ER-stress by inhibiting glutathione synthesis leading to excessive free radicals production and activating ER-stress pathways. Hence glutamate-induced toxicity plays a role in the pathogenesis of various diseases such as Huntington’s, amyotrophic lateral sclerosis (ALS), Alzheimer’s, and Parkinson’s, it is thought that protection of neuronal cells against glutamate-induced excitotoxicity can be an effective therapeutic approach against the aforementioned neurodegenerative diseases.

Thiamine, also known as vitamin B1 because it is the first vitamin to be discovered, is found in foods such as whole grains, legumes, and fish. It also manufactured for dietary supplements and medical therapy. Thiamine is an essential micronutrient because it could not be synthesis in the body. In addition, thiamine is needed to sustain glucose, amino acids, and lipids metabolism in physiological conditions. Moreover, thiamine deficiency leads to disorders, including beriberi and Wernicke encephalopathy.

On the other hand, because of its high metabolic capacity, the brain is one of the most undefended organs to thiamine deficiency. Furthermore, it has been found that thiamine supplementation or pretreatment is useful for nervous system disorders as well as thiamine deficiency. Moreover, it has been found that thiamine pretreatment protects neuronal damage after glutamate-induced excitotoxicity. However, the mechanisms underlying gliotoxicity caused by glutamate are still not clear enough. Here we examined the protective effects of thiamine on glutamate-induced cell death, which is a gliotoxicity model, using glioblastoma (C6) cell cultures.

Regular Article

Thiamine Protects Glioblastoma Cells against Glutamate Toxicity by Suppressing Oxidative/Endoplasmic Reticulum Stress

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Thiamine (vitamin B1), which is synthesized only in bacteria, fungi and plants and which humans should take with diet, participates in basic biochemical and physiological processes in a versatile way and its deficiency is associated with neurological problems accompanied by cognitive dysfunctions. The rat glioblastoma (C6) model was used, which was exposed to a limited environment and toxicity with glutamate. The cells were stressed by exposure to glutamate in the presence and absence of thiamine. The difference in cell proliferation was evaluated in the XTT assay. Oxidative stress (OS) markers malondialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT) levels, as well as endoplasmic reticulum (ER) stress markers 78-kDa glucose-regulated protein (GRP78), activating transcription factor-4 (ATF-4), and C/EBP homologous protein (CHOP) levels, were measured with commercial kits. Apoptosis determined by flow cytometry was confirmed by 4’,6-diamidino-2-phenylindole (DAPI) staining. At all concentrations, thiamine protects the cells and increased the viability against glutamate-induced toxicity. Thiamine also significantly decreased the levels of MDA, while increasing SOD and CAT levels. Moreover, thiamine reduced ER stress proteins’ levels. Moreover, it lessened the apoptotic cell amount and enhanced the live-cell percentage in the flow cytometry and DAPI staining. As a result, thiamine may be beneficial nutritional support for individuals with a predisposition to neurodegenerative disorders due to its protective effect on glutamate cytotoxicity in glioblastoma cells by suppressing OS and ER stress.

Key words thiamine; glutamate; oxidative stress; endoplasmic reticulum stress; C6 rat glioma
Experimental

Cell Culture and Treatment The C6 (CRL107) rat glioma cells was utilized in this research because it composes a suitable model of glutamate-mediated cytotoxicity.17) The cells were routinely grown in Dulbecco’s modified Eagle’s medium (DMEM) consist of 1% pen-strep, and 10% fetal bovine serum (FBS) at 37°C within 5% CO₂ humidified atmosphere. Thiamine and glutamate were dissolved in physiological saline and freshly prepared just before use on the experiment days. Four different cell groups were determined to assess the possible neuroprotective effect of thiamine. Our first group was the control group in which no drug solution was administered. Cells in the second group, the glutamate group, were exposed for 24 h to the concentration of glutamate (10 mM) used to model the gliotoxicity. The cells in the third group (thiamine group) were healed with increasing (0.025, 0.05, 0.1, 0.2, and 0.4 mg/mL) concentrations of thiamine for 24 h. And finally, cells in our fourth group (thiamine + glutamate group) were treated with increasing concentrations (0.025, 0.05, 0.1, 0.2, and 0.4 mg/mL) of thiamine for 1 h, and then 10 mM glutamate was added to each well and exposed for 24 h.

Cell Viability Assay Thiamine was tested for protective activity against glutamate on C6 cells using the XTT assay (Abcam, U.K.) following the reduction of the XTT-tetrazolium salt to an XTT-formazan product (orange-colored). Cells were disseminated at $1 \times 10^4$ cells/well into the 100μL medium and wait for attachment, following which they were handled with thiamine (0.025, 0.05, 0.1, 0.2, and 0.4 mg/mL) for 1 h before glutamate treatment. The day after, a 50μL mixture of XTT reagents was added to cultured cells in each well of a 96-well plate, which was then incubated at 37°C for 4 h. The produced formazan crystals were dissolved in the culture medium, and absorbance were measured by spectrophotometry using a microplate reader (Multiskan PLUS, Thermo Scientific, MA, U.S.A.) at 450 nm. All tests were repeated in triplicate and cell viability was evaluated as a percentage of viable cells against the control group, which were untreated cells.

Preparation of Cells for Enzyme-Linked Immunosorbent Assay (ELISA) Tests The untreated and treated cells were collected in a sterile tube and centrifuged at 2000rpm for 10min. After the upper phase were deported, cell pellets were diluted through phosphate-buffered saline to a cell concentration of 1 million/mL. The cell membrane was fragmented by repeated freeze-thaw and intracellular components were allowed to withdraw. The obtaining mixture was centrifuged at 4000rpm for 10 min and the supernatants were collected in different sterile tubes to perform biochemical analysis. The total protein level contained in each tube was determined with the Bradford Assay Kit (Abcam, U.K.).

Measurement of Oxidative and Endoplasmic Reticulum Stress Markers, and Caspase-3 Levels in the Cells The malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), 78-kDa glucose-regulated protein (GRP78), activating transcription factor-4 (ATF-4), C/EBP homologous protein (CHOP), and caspase-3 levels of the obtained cell lysates were estimated using commercial ELISA kits (YL Biont, Shanghai, China). The assay protocol was carried out following the steps written in the user manual. The absorbance values resulting from the color change of standard, untreated and treated cell samples at the end of the incubation period were evaluated at 450nm in an ELISA reader (Thermo Fisher Scientific, Altrincham, U.K.). The values of the samples were determined by drawing the standard curve graphics. It was determined that the inside and between the plates have less than 10% variation coefficients.

Annexin V Binding Assay Apoptosis was assessed by utilizing the Muse Annexin V & Dead Cell (Luminex, Tokyo, Japan) assay kit. Six different cell groups were determined as control, thiamine (0.025 and 0.4 mg/mL), glutamate (10mM) and thiamine (0.025 and 0.4mg/mL) + glutamate (10mM). After the cells were attached to the plate base, drug solutions were applied under conditions suitable for glutamate toxicity and incubated for 24h. The apoptosis detection test was enforced in line with the instructions of the user manual guide and measurements were made in the Guava® Muse® Cell Analyzer in accordance with the report in our previous study.18)

4',6-Diamidino-2-phenylindole (DAPI) Staining After 24h of exposure to glutamate and/or 1-h pretreatment with...
thiamine, the cells were fixed with paraformaldehyde (4%) for 10 min at 21 °C. Cells were scrubbed three times with phosphate buffered saline (PBS), then tinged with DAPI (BioShop, Burlington, Canada) for 5 min, and inspected with an Olympus BX51 fluorescence microscope. Considering that decreased nuclear size, nuclear fragmentation, chromatin condensation, and intense fluorescence represented apoptosis, apoptosis-related alterations in nuclear morphology were inspected. Cells were visualized using Olympus software.

**Statistical Analysis** Statistical analysis was performed by one-way ANOVA and post hoc Tukey test by comparing control with thiamine treated cells at different concentrations using SPSS Version 23.0 for Windows.

**Results**

**Effects of Thiamine Administration on Glutamate-Induced C6 Cell Cytotoxicity** The XTT cell viability kit was carried out to evaluate the effects of thiamine against glutamate-mediated gliotoxicity in C6 cells. In this study, it was tested that the effect of increasing doses of thiamine (0.25–4 mg/mL) on cell viability in both control and glutamate-exposed C6 cells. The cells were initially treated with increasing concentrations of thiamine (0.25, 0.5, 1, 2, and 4 mg/mL) for 60 min and then incubated absence or presence glutamate (10 mM) for 24 h. As shown in Fig. 1, 24-hour exposure of C6 cells to glutamate notably reduced the viability of the cells compared to the control group \( p < 0.001 \); Fig. 1). However, the thiamine concentrations tested enhanced cell survival compared to glutamate-exposed C6 cells \( p < 0.001 \); Fig. 1). Also, thiamine at all concentrations did not alter C6 survival compared to untreated cells \( p > 0.05 \); Fig. 1).

**Effect of Thiamine Pretreatment on MDA, SOD, and CAT after Glutamate-Induced Gliotoxicity** The ELISA assays were carried out to evaluate the effects of thiamine on oxidative stress parameters after glutamate-mediated cytotoxicity in the glioblastoma cells. The C6 cells were incubated for 24 h in the presence or absence of glutamate (10 mM) after being treated with the lowest and highest concentration of thiamine (0.25 and 4 mg/mL) for one hour. Preincubating the C6 cells with glutamate for 24 h impressively increased MDA levels and decreased the SOD and CAT levels against the control group \( p < 0.001 \); Fig. 2). However, thiamine (0.25 and 4 mg/mL) significantly reduced MDA levels and raised SOD and CAT levels in C6 cells against the glutamate-treated C6 cells \( p < 0.001 \); Fig. 2).

**Effect of Thiamine Pretreatment on GRP78, ATF-4, CHOP, and Caspase-3 after Glutamate-Induced Cytotoxicity in the C6 Cells** The effects of thiamine on ER stress proteins and caspase-3 were evaluated for glutamate-induced cytotoxicity in C6 cells using ELISA kits. Cells were treated with thiamine (0.25 and 4 mg/mL) for 1 h and then incubated with/without glutamate (10 mM) for the next 24 h. Twenty-four-hour pre-incubation of C6 cells with glutamate significantly increased the levels of GRP78, ATF-4, CHOP, and caspase-3 compared to untreated control cells \( p < 0.001 \); Fig. 3). However, thiamine (0.25 and 4 mg/mL) significantly reduced GRP78, ATF-4, CHOP, and caspase-3 levels in C6 cells against the glutamate-exposed C6 cells \( p < 0.001 \); Fig. 3).

**Effect of Thiamine Pretreatment on Apoptosis after Glutamate-Mediated Cytotoxicity in the C6 Cells** The anti-apoptotic effects of thiamine on the gliotoxicity model generated in C6 cells were determined by the flow cytometry method. The results of flow cytometry analysis were shown in Fig. 4, and it is displayed that glutamate treatment exceptionally increased the ratio of apoptotic cells for 24 h against the control (without glutamate) cells \( p < 0.001 \); Fig. 4). Furthermore, thiamine (0.25 and 4 mg/mL) pretreatment remarkably decreased the apoptotic ratio in the gliotoxicity model \( p < 0.001 \); Fig. 4). Nevertheless, treating C6 cells with thiamine (0.25 and 0.4 mg/mL) alone did not show any considerable change in apoptotic effect \( p > 0.05 \); Fig. 4).

**Effects of Thiamine on Nuclear Changes after Glutamate-Induced Cytotoxicity in the C6 Cells** Alterations in nuclear
morphology were investigated in the control, glutamate-treated (10 mM), thiamine (0.25 and 0.4 mg/mL) pre-treated with glutamate, and thiamine pre-treated without glutamate groups by DAPI staining (Fig. 5). The control cell nuclei were well ordered in shape. Morphological presentations of apoptosis were monitored in cells treated with glutamate, including reduced nuclear size, chromatin condensation, nuclear fragmentation, and intense fluorescence. These changes in nuclear morphology were mitigated by pretreatment of cells with thiamine (0.25 and 0.4 mg/mL) after glutamate-induced cytotoxicity in cells.

**Discussion**

In the present study, it has been evaluated that the effect of thiamine pretreatment against cytotoxicity caused by glutamate in the glioblastoma (C6) cells. Pretreatment with thiamine raised cell viability and decreased cell death after glutamate-induced cytotoxicity in the C6 cells. Moreover, thiamine pretreatment lessened MDA levels and increased SOD and CAT levels in the C6 cells. Besides, thiamine pretreatment suppressed the ER-stress pathway proteins, GRP78, ATF-4, and CHOP, after glutamate-induced cytotoxicity in the C6 cells.

The present study proposed that thiamine can inhibit glutamate-induced apoptotic cell death in C6 cells. Thiamine, an essential micronutrient, can easily pass through the biological membrane and blood–brain barrier, involved in glucose metabolism, the maintenance of nerve membrane function, and the synthesis of myelin and several types of neurotransmitters. Because of these beneficial effects, thiamine has been applied as a therapeutic agent for treating various diseases, including neurological disorders.

Astroglial cells are known to be able to support and promote neuronal survival, and therefore the damage of these cells may increase neuronal loss. Recent evidences report that astroglial dysfunction is closely linked to several neurological diseases, such as Alzheimer’s disease, Parkinson’s disease, and ischemic stroke. C6 rat glioblastoma cells are frequently used as a model appliance to investigate astroglial cell function and excitotoxicity due to their characteristics of expressing ionotropic and metabotropic glutamate receptor subtypes as well as glial fibrillar acidic protein and S100B. The in vitro model toxicity concentration of glutamate, which has been shown to trigger excitotoxicity in C6 cells depending on the dose and time, varies between 0.01 and 20 mM according to the experimental culture conditions. 10 mM glutamate was used in our study. Preliminary studies were conducted to determine the optimal glutamate concentration before cells were treated to determine the concentration. In this context, after the glutamate concentrations in the range determined in accordance with the literature were applied to the cells, the cytotoxic concentration was determined in half of the cells.

Recent findings have shown that thiamine has beneficial effects on the nervous system. It has been shown that thiamine protects murine cerebellar granule cells from glutamate/N-methyl-D-aspartate (NMDA) toxicity. Moreover, it has been pointed out that thiamine antagonists trigger p53-dependent apoptosis in differentiated SH-SY5Y neuroblastoma cells and induce apoptosis in the different types of nerve cell lines such as rat PC-12 and rat astrocytes DITNC cell lines. However, in contrast to these studies, it has been claimed that thiamine does not affect glutamate-induced neurotoxicity in retinal cultures. In the present study, thiamine pretreatment in all doses alleviated glutamate-induced cytotoxicity in rat C6 glioma cell line similar to previous studies.

Oxidative stress (OS) means an imbalance between oxidants and antioxidant defense systems in the organism. The imbal-
ance in the oxidants and antioxidant systems causes excessive reactive oxygen species (ROS) generation, which gives rise to harms tissues and gums up to the physiological function of the organism. Moreover, it has been shown that OS is one of the most important risk factors of neurodegenerative diseases and CNS disorder. Previous studies have reported that thiamine protects tissues from oxidative damage by increasing antioxidant enzymes, CAT and SOD, and reducing lipid peroxidation. Consistent with these studies, in this study thiamine pretreatment increased antioxidant enzymes, SOD and CAT, levels and also decreased MDA levels, which is a marker of oxidative damage after glutamate exposed cytotoxicity in the C6 cells.

The ER is the most important organelle in which protein folding is regulated in eukaryotes. In physiological or pathological conditions that exceed the ER functional capacity, unfolded or misfolded protein accumulation occurs in the ER lumen, which is called ER stress. After ER-stress occurs, a series of events called unfolded protein response (UPR), consisting of a number of intracellular signaling pathways such as protein kinase the GRP78, activating transcription factor 6 (ATF-6), protein kinase RNA (PKR)-like ER kinase (PERK), and inositol-requiring enzyme-1 (IRE1), are activated in order to restore homeostasis in the cell and get rid of stress with minimal damage. The activation of GRP78 and PERK induces cell signaling of eukaryotic initiation factor 2 alpha

Fig. 4. Effect of Thiamine on Apoptosis in C6 Cells after Glutamate-Induced Cytotoxicity
The data are expressed as mean ± standard error mean. *** p < 0.001 as compared to control-untreated group; ### p < 0.001 compared to glutamate-treated group.
The activation of CHOP in the final step leads to exogenous-induced apoptosis in the cell by increasing caspase-3 levels. Moreover, glutamate-induced cytotoxicity induces ER-stress leading to neuronal apoptosis. Furthermore, it has been exhibited that glutamate-induced cytotoxicity in the C6 glioma activates ER-stress pathways. Besides, it has been demonstrated that thiamine deficiency induces ER-stress in neurons. In the present study, glutamate-induced cytotoxicity increased ER-stress related proteins (GRP78, ATF-4, and CHOP) and also caspase-3 levels. However, thiamine pretreatment reorganized the ER-stress and caspase-3 levels in the C6 glioma after glutamate-induced cytotoxicity.

Characterized by some morphological and biochemical differences in the neuronal cells in which they occur, apoptosis and necrosis play a key role in the pathogenesis of many nervous system related disorders. In fact, apoptosis is generally accepted to occur by activating the neurotoxic effects of glutamate, a stimulating neurotransmitter of the central nervous system, through extracellular factors. The prolonged presence of glutamate in the intercellular space has extensive consequences on neuron functioning, starting with an increase in intracellular Ca concentration, an imbalance of transmembrane gradients of major electrogenic ions, and activation of various intracellular cascades and ending with destruction of the plasma membrane. In the present study, glutamate induced not only apoptosis but also necroptosis in the C6 cells according to the cytometry. Thiamine suppressed both apoptosis and necroptosis after glutamate-induced cytotoxicity. However, Park et al. have been shown that glutamate activates only apoptosis not necroptosis in the C6 cells after glutamate-induced gliotoxicity according to Hoechst dye 33342 and propidium iodide (PI) in contrast to our study. Nevertheless, it is not correct to claim that necrosis does not activate after glutamate-induced gliotoxicity on the basis of PI staining alone. In addition, it has been suggested that glutamate-induced cytotoxicity activates also necroptosis pathway as well as apoptosis in the neural cells. This discrepancy could be related to methodological differences between the studies to find out about apoptosis or necroptosis. However, our findings are not enough to explain discrepancy deeply between studies and need further investigations.
stress and ER-stress (Fig. 6). A problem that may occur in the biochemical and physiological processes of the glial cells can bring along neurological problems. The lack of thiamine required by humans in the diet is also associated with neurodegeneration-related diseases accompanied by cognitive dysfunction and may be a supportive therapeutic agent. However, it is needed to be demonstrated by further investigations.

The study has potential limitations. It is necessary to examine the effects of antagonists such as amprolium and oxythiamine on the protective effects of thiamine. However, thiamine antagonists’ effects on glutamate-induced cytotoxicity is missing in the present study. This situation is a limitation of the present study. Moreover, total oxidant status (TOS) levels in the group after glutamate-induced cytotoxicity were measured using spectrophotometric method to determine oxidative stress. Nonetheless, it is better to measure directly intracellular ROS levels by using fluorometric method. This is another limitation of the current study.

Conclusion

The results of this study represented that thiamine decreased cell death after glutamate-induced cytotoxicity in C6 cells. This protective effect may consist through the inhibition of oxidative stress and ER-stress pathways. Thereupon, thiamine supplementation could be protective in CNS disorder and chosen as a useful therapeutic agent in neurodegenerative related disorders. Due to the limited study budget, the possible protective effect of thiamine has been investigated, and it is aimed to use thiamine antagonists to investigate the mechanisms underlying the neuroprotective effect of thiamine with further studies. However, more research is needed to answer questions about possible mechanisms.

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Conflict of Interest

The authors declare no conflict of interest.

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