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The neuroprotective effect of lamotrigine against glutamate excitotoxicity in SH-SY5Y human neuroblastoma cells

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ABSTRACT

Objective: Glutamate-induced excitotoxicity has a role in the pathophysiology of neurodegenerative disorders. Lamotrigine, an antiepileptic drug, also used to treat bipolar disorders, may be protective against excitotoxic insult. The aim of the study was to investigate the neuroprotective effect of lamotrigine against the glutamate excitotoxicity in SH-SY5Y cell line.

Materials and Methods: SH-SY5Y human neuroblastoma cells were pre-treated with lamotrigine (50-100-150 μM) prior to exposure to 15 mM glutamate. The 3-(4,5-dimethythiazol – 2-yl)-2,5 – diphenyl tetrazolium bromide (MTT) assay was performed to determine cell viability. The anti-oxidant effect of lamotrigine and the role of inflammatory parameters were determined by measuring superoxide dismutase (SOD), hydrogen peroxide (H_2O_2), IL-1β, IL-6 and TNF-α.

Results: Intracellular calcium levels and lactate dehydrogenase (LDH) activity increased in glutamate exposed cells. Pre-treatment of cells with MK-801 showed no protective features against glutamate excitotoxicity. Treatment with 100 μM lamotrigine was effective in increasing the viability of glutamate exposed cells and in reducing H_2O_2 increase in these cells. The SOD activity increased by lamotrigine treated cells exposed to glutamate. IL-1β, IL-6 and TNF-α levels increased after induction with glutamate and attenuated by lamotrigine.

Conclusion: Overall, our results confirmed the critical role of inflammation and oxidative stress in glutamate-induced excitotoxicity and lamotrigine may exert a protective effect.

Keywords: Lamotrigine, Glutamate excitotoxicity, SH-SY5Y, Oxidative stress parameter, Cytokines, MK-801

1. INTRODUCTION

Lamotrigine is an anticonvulsant drug used in the treatment of bipolar disorder. It has a modest effect in depressive episodes and its benefit in maintenance therapy is more effective when combined with other mood stabilizers such as lithium or valproate [1,2].

As, lamotrigine has a broad anticonvulsant spectrum and psychotropic profile, it is proposed to have distinctive cellular effects that contribute to its broad effects. Its well-demonstrated cellular mechanism of action is the blockade of neuronal voltage-gated sodium channels and subsequent stabilization of neuronal membranes and suppression of post-synaptic glutamate release [3].

The disruption of normal excitatory neurotransmission regulated by glutamate and its ligand-gated ionotropic glutamate receptors are involved in a wide range of pathophysiology of neurological conditions such as epilepsy [4], hypoxic-ischemic brain damage [5] and neurodegenerative disorders such as Alzheimer's, Parkinson's, Huntington's disease and multiple sclerosis [6].

The involvement of abnormal Na^+ influx and voltage-gated sodium channel activity in the pathophysiology of these disorders lead to the investigation of neuroprotective effects of lamotrigine. Hence, it is effective in status epilepticus [7], oxygen-glucose deprivation [8], neonatal hypoxic-ischemia [9] and adult ischemia [10]. Despite its widespread clinical use and accepted neuroprotective effects, the molecular mechanisms of its therapeutic actions need to be identified.

Chronic neuroinflammation plays an important role in the pathogenesis of neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease and multiple sclerosis [11]. It is also known that these diseases develop in older age

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and normal aging process induces several cellular changes including increase in intracellular Ca^{2+} levels which causes low-grade inflammation in the central nervous system (CNS) and the peripheral systems [12]. This low-grade inflammation is associated with increase in inflammatory mediator release such as interleukin-1beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α).

Thus, regulating pro-inflammatory mediators in neurodegenerative disorders may have the therapeutic potential to reduce neuronal injury in neurodegenerative diseases. Infection or inflammation that occurs in response to injury, is associated with several neurotoxic and pro-inflammatory mediators. These mediators are reactive oxygen species (ROS), nitric oxide, prostaglandin E_2, as well as pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α. Accordingly, we investigated the neuroprotective effect of lamotrigine against glutamate toxicity in the SH-SY5Y cell line by measuring inflammatory and oxidative system parameters.

2. MATERIALS and METHODS

Cell culture model

The study was conducted using the SH-SY5Y human neuroblastoma cell line. The SH-SY5Y cells were grown in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific Inc., UK) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific Inc., UK), 100 IU/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific Inc., UK). The cells were seeded into 96-well plates ($1x10^4$ cells/well) and cultured in an atmosphere of 5% CO_2 and saturated humidity at 37.0˚C. SH-SY5Y cells were incubated in complete culture medium for 24 h prior to the addition of glutamate or lamotrigine regarding the investigation of the effects of glutamate and lamotrigine.

Drug concentrations

Cells were treated with different concentrations of glutamate (1-50 mM; L-Glutamic acid; cat. no. G1251; Sigma-Aldrich, USA) in order to determine the glutamate toxicity in the cultured SH-SY5Y cells. 15 mM glutamate produced a significant decrease in cell viability, ~20% of control after 24 h. Subsequently, 15 mM was selected as the working concentration of glutamate to be used in the following experiments.

MK-801 was purchased from Sigma-Aldrich Co. (cat. No. M107, Germany) and used to investigate the role of N-methyl-D-aspartate (NMDA) receptor in glutamate toxicity. MK-801 (1, 5, or 10 µM) was added to culture medium 3 h before and co-incubated with 15 mM glutamate for 24 h.

The SH-SY5Y cells were treated with lamotrigine (Sigma-Aldrich Co. cat. No. L3791, Germany), concentrations of 50 µM, 100 µM, 150 µM 1 h prior to glutamate exposure. Lamotrigine was dissolved in 12 mg / ml dimethyl sulfoxide. As 100 µM was found to be the neuroprotective concentration of lamotrigine, this concentration was used in the following experiments.

Cell viability assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay kit (Thermo Fisher Scientific Inc., USA) was used to evaluate cell viability in line with the manufacturer's instructions. After adding MTT solution (5 mg/ml) to each well, cells were incubated for 3 h with 5% CO_2 at 37˚C. Following the removal of the culture medium, 200 µl dimethyl sulfoxide was used to dissolve the formazan product. Absorbance values were measured at 560 nm wavelength using a microplate reader (Multiskan™ GO microplate spectrophotometer; Thermo Fisher Scientific Inc., Finland). Cell viability was calculated by considering the controls as 100%.

Cell lysate preparation

SH-SY5Y cells were harvested by using Trypsin-EDTA 0.25% (Thermo Fisher Scientific Inc., UK) and collected by centrifugation at 1,000-2,000 x g for 10 min at 4˚C. Onwards, cells were then re-suspended with ice-cold buffer (0.05 M potassium phosphate, pH 7.0, 1 mM EDTA) then homogenized by sonication on ice. The lysate was centrifuged at 12,000 x g at 4˚C for 20 min to remove cell debris. The supernatant was further used for determining the quantity of total protein and for the enzyme activity assay. The protein concentration was determined using the bicinchoninic acid assay kit (Thermo Fisher Scientific Inc., USA). All spectrophotometric measurements were made using an Epoch microplate spectrophotometer (BioTek Instruments, Inc., USA).

Intracellular calcium concentration ([Ca2]i) assay

Intracellular Ca^{+2} measurement was performed using Ca^{+2} colorimetric assay kit (CAT No:K380; Biovision Inc., USA). The kit utilizes the chromogenic complex (λ = 575 nm) formed between calcium ions and 0-cresolphthalein and calcium concentration determined by means of a standard curve generated using calcium standard (500 mM). All samples, standards and controls were measured in duplicate.

Lactate dehydrogenase (LDH) assay

The LDH cytotoxicity was determined in the prepared supernatant with an enzyme linked immunosorbent assay (ELISA) kit (Pierce LDH Cytotoxicity Assay Kit; Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions.

Cytotoxicity was calculated with the following equation;

Cytotoxicity (%)=(Sample LDH activity – Spontaneous LDH activity)/(Maximum LDH activity – Spontaneous LDH activity) x 100

IL-1β, IL-6, TNF-α assay

The concentrations of IL-1β, IL-6 and TNF-α were determined using ELISA kits (Invitrogen BMS224HS, KHC0061, BMS223HS; Austria). According to the manufacturer's instructions, a colored product was formed in proportion to the amount of cytokines present in the sample or standard. The reaction was terminated by

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addition of acid and absorbance was measured at 450 nm. A standard curve was prepared from standard dilutions for each cytokine and sample concentration was determined. All of the samples, standards and controls were estimated using duplicate analyses.

Superoxide dismutase (SOD) activity assay.

The SOD assay was performed by quantifying the inhibition of nitro blue tetrazolium (NBT) at 560 nm wavelength. The assay mixture (200 µl) comprised of 0.0033 mM riboflavin, 10 mM L-methionine, 0.033 mM NBT and 0.66 mM EDTA-Na_2 in 0.05 M potassium phosphate buffer (pH 7.8). The 96-well plates containing the assay mixture were incubated for 20 min with 300 nmol/m^2/sec at 560 nm excitation at 25˚C. One unit of SOD activity was defined as the amount of protein (mg) causing 50% inhibition of photoreduction, following which specific enzyme activity was expressed as units/mg protein.

Hydrogen peroxide (H_2O_2) activity assay

The H_2O_2 activity in cells were quantified using H_2O_2 assay kit (cat no. ab102500; Abcam, USA). At the 24th h after drug administration, cells were harvested, homogenized and centrifuged. The supernatant was used for the assay. The absorbance was detected at 570 nm using a microplate reader and the optical density was used for quantification of H_2O_2 levels. Distilled water was used as a negative control instead of a cell lysate sample.

Statistical Analysis

Values are expressed as the mean ± standard error of the mean and analyzed by one-way analysis of variance (ANOVA) followed by a Tukey's multiple-comparisons post-hoc test. A P value <0.05 was regarded as a statistically significant difference.

3. RESULTS

There was a significant decrease in cell viability in 15 mM glutamate exposed cells without any treatment (Figures 1 and 2). As shown in Figure 1, MK-801 pretreatment in three different doses have not protected cells from glutamate-induced excitotoxicity. MK-801 alone did not exhibit any significant effect on the cell's activity.

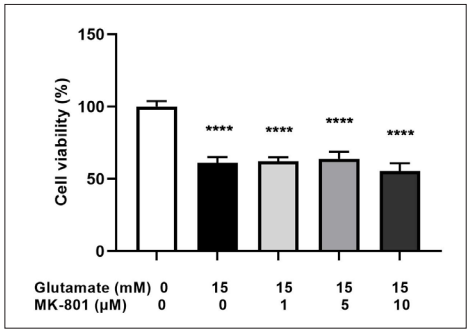

Figure 1. *Figure indicating the 15 mM glutamate-induced decrease in cell viability in SH-SY5Y cells as % of control. Cells were treated with 3 different concentrations of MK-801 (1, 5, or 10 µM). Cell viability (% of control) is expressed as the mean value of four separate experiments. ****P<0.0001 vs. control*

After cells were treated with lamotrigine, the cell viability was enhanced, and the protective effect of lamotrigine on cells after glutamate exposure was significant at a concentration of 100 µM (Figure 2).

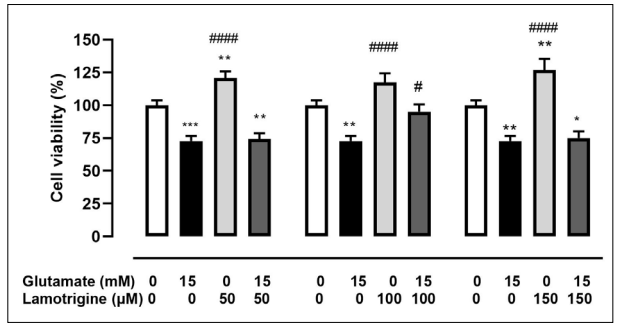

Figure 2. *Effect of lamotrigine pre-treatment on the viability of SH-SY5Y cells with glutamate-induced excitotoxicity. Viability of SH-SY5Y cells pre-treated with or without three different lamotrigine concentrations (50, 100, 150 µM) followed by exposure to 15 mM glutamate to induce excitotoxicity. Cell viability (% of control) is expressed as the mean value of four separate experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs control group; # P<0.05, #### P<0.0001 vs glutamate group*

Intracellular calcium levels in glutamate exposed cells were detected to be increased when compared to control cells that were not exposed to glutamate (P<0.05 vs control group). Lamotrigine significantly inhibited glutamate-induced elevation of [Ca^{2+}]I (P<0.005 vs glutamate group; Figure 3A).

The LDH activity increased with glutamate cytotoxicity and decreased significantly below the control levels in lamotrigine pre-treated cells that were exposed to glutamate (P<0.05; P<0.005 vs control group; Figure 3B).

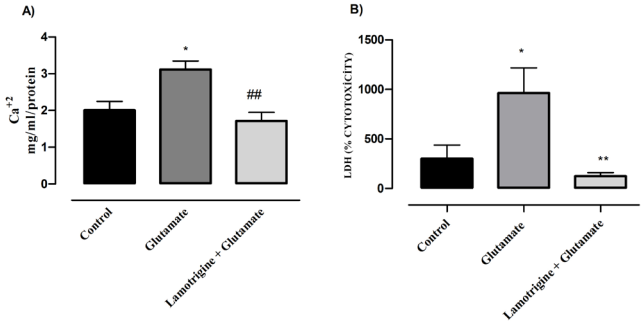

Figure 3. *(A) Ca^{2+} and (B) LDH levels in lamotrigine-pre-treated SH-SY5Y cells exposed to glutamate to induce excitotoxicity. *P<0.05 vs. control group; ##P<0.01 vs. glutamate group in A. *P<0.05, **P<0.01 vs. control group in B. Values are expressed as the mean value of four separate experiments.*

The IL-1β, IL-6 and TNF-α levels increased significantly after induction with glutamate. Lamotrigine application attenuated these increased levels induced by glutamate (Figure 4).

The SOD activity slightly increased after glutamate exposure, but this alteration was not statistically significant. The SOD

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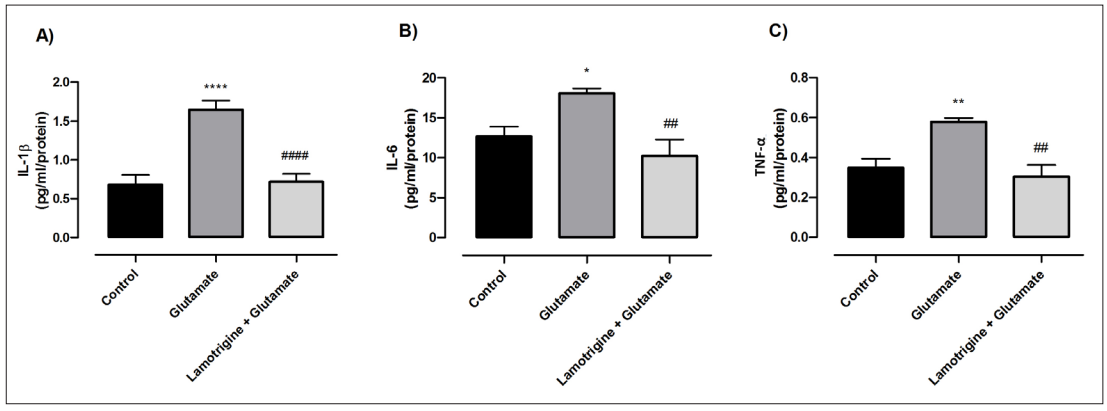

Figure 4. *(A) Interleukin-1β (IL-1β), (B) Interleukin-6 (IL-6) and (C) Tumor necrosis factor – α (TNF – α) levels in lamotrigine-treated cells exposed to glutamate. ****P<0.0001 vs control group; #### P<0.0001 vs glutamate group in A. *P<0.05 vs. control; ##P<0.01 vs. glutamate group in B; **P<0.01 vs control group; ## P<0.01 vs glutamate group in C.*

activity increased in the lamotrigine pre-treated cells when exposed to glutamate. This increase was significant as compared with that in the control group and glutamate group (P<0.001; P<0.01, respectively; Figure 5A).

After 24 h of incubation with 15 mM glutamate, H_2O_2 levels increased as compared with those in the control group (P<0.0001). Treatment with lamotrigine was indicated to decrease H_2O_2 levels in cells exposed to glutamate when compared to treatment with glutamate alone (P<0.0001), and data revealed that it was able to reduce it to the control levels (Figure 5B).

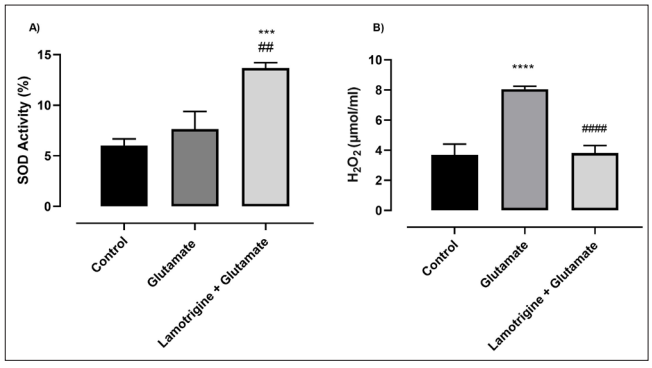

Figure 5. *(A) SOD activity and (B) H_2O_2 in control cells, lamotrigine-treated cells, cells exposed to glutamate, as well as lamotrigine-pre-treated SH-SY5Y cells exposed to glutamate excitotoxicity. ***P<0.001 vs control group; ## P<0.01 vs glutamate group in A; ****P<0.0001 vs control group; #### P<0.0001 vs glutamate group in B. SOD, superoxide dismutase; H_2O_2, hydrogen peroxide*

4. DISCUSSION

Cell viability analysis was used as an indication of cell death to determine the toxic glutamate dose for SH-SY5Y cell lines. We demonstrated that pre-treatment with 100 μM lamotrigine significantly inhibited cell toxicity caused by glutamate as a measurement of cell viability. The results of cytotoxicity due to LDH release also supported this finding. The neuroprotective effect of lamotrigine was investigated in several in-vivo models and it was shown that 10 and 20 mg/kg lamotrigine had protective effect in vivo model of neonatal hypoxic-ischemic encephalopathy in rats [9]. In the study of Halonen et.al., 12.5 mg/kg, twice a day lamotrigine treatment demonstrated reduction in the duration or severity of the status epilepticus, showing mild neuroprotective effect observed in the hippocampus and piriform cortex of rats [7]. Walker et al., reported that the mean serum concentration was 23.03±2.1 μM with 10 mg/kg and 46.6 ±7.7 μM with 20 mg/kg administration of lamotrigine [13]. However, similar to our results, Leng et al., revealed that 100 μM lamotrigine had a neuroprotective effect in glutamate-induced primary neuronal cerebellar granule cells via histone deacetylases (HDAC) inhibition and up-regulation of anti-apoptotic Bcl-2 [14]. Also, the dose used in the present study was comparable to the lamotrigine's therapeutic target range of 10–60 μM in the serum [15].

We found that MK-801, a NMDA receptor antagonist was not effective in reversing cell viability which was decreased by glutamate administration suggesting that the cytotoxic effect of glutamate in SH-SY5Y cells was related with any other mechanism rather than NMDA receptor. The previous studies showed controversial results that the effect of glutamate on SH-SY5Y cells was whether NMDA-mediated or not [16-19]. The expression of metabotropic and ionotropic receptors was shown in this cell line by some studies [16,17] whereas, others revealed that NMDA receptors were not expressed in SH-SY5Y cells, suggesting the increase of cytoplasmic Ca^{+2} was independent of glutamate receptors [18,19]. Glutamate toxicity could be explained in two forms, one was receptor-related excitotoxicity [20] and the other was non-receptor related oxidative glutamate toxicity [21]. It was reported that after glutamate exposure, calcium influx caused induction of free radical generation from mitochondria and these free radicals could cause cell membrane peroxidation and might activate inflammation signaling pathways with cell damage, and might also disrupt

the blood–brain barrier by affecting the endothelial basement membrane [22].

We determined that intracellular calcium levels increased in glutamate exposed cells. This finding was in accordance with the other reports revealing that the Ca^{2+} dependent release of glutamate involved intracellular Ca^{2+} stores in astrocytes [23,24]. It is speculated that sustained Ca^{2+} influx through glutamate receptor channels was an important pathway of neuronal cell death. The increase of glutamate levels in the CNS might cause elevated intracellular Ca^{2+} levels, which lead to a rise in the Ca^{2+} concentration in sensitive organelles such as mitochondria and the endoplasmic reticulum [25]. It was accepted that the sustained high levels of intracellular Ca^{2+} subsequent to Na^{+} or both, might lead to neuronal degeneration involving several different pathways that caused oxidative stress and degeneration [21,26]. We demonstrated that lamotrigine decreased the elevated Ca^{2+} levels due to glutamate exposure. In accordance with this finding, it was reported that lamotrigine, besides its action on voltage-dependent sodium channels, also affected the neuronal calcium channel, and calcium antagonistic actions were discussed to have a role in treatment strategies of epilepsies [27].

Glutamate, a major excitatory neurotransmitter in CNS, may lead to the development of various neurodegenerative diseases when present at high concentrations by inducing oxidative stress and neurotoxicity. Oxidative stress has an important role leading to neuronal loss and death. Glutamate-induced cell death involves the inhibition of glutathione synthesis and depletion and causes excessive ROS production resulting in oxidative stress. The accumulation of excessive ROS can cause functional and structural changes in the mitochondria and activate cell death pathways [21,28].

We evaluated the levels of SOD and H_2O_2 to determine the role of oxidative stress on glutamate-induced cell death in SH-SY5Y cells. H_2O_2, can readily cross the cell membrane, affects cellular structure distant from its origin and is considered most suitable for redox signaling among the various oxygen metabolites [29]. We found a significant increase in H_2O_2 levels after acute exposure of 15 mM glutamate in SH-SY5Y neuroblastoma cells suggesting that glutamate-induced excitotoxicity was mediated with oxidative stress. The studies suggesting the contribution of oxidative stress in glutamate excitotoxicity also presented similar findings with our study. Ha et al., stated an accumulation of extracellular H_2O_2 after prolonged exposure to glutamate in a time – and concentration-dependent manner in HT22 cells [30]. It was reported that glutamate at 1-50 mM concentration affected H_2O_2 synthesis by brain mitochondria and this effect was associated with complex II, a source of superoxide anion formation in mitochondria and was dependent on the mitochondrial potential [31]. We found a non-significant increase in SOD activity which was not consistent with other studies that had reported a decrease with glutamate [18,32]. ROS is converted to less reactive hydrogen peroxide by SOD through the use of copper/zinc or manganese and could play a protective role in neurodegeneration and had been thought to be activated firstly for defense systems against oxidative stress, in neurodegeneration [33]. We think that a protective mechanism was activated as a response to increased ROS after glutamate exposure as mentioned that increase of SOD was related to the adaptive mechanism against the raised amount of lead-induced ROS production [34].

We also determined that pre-treatment with lamotrigine caused an increase in SOD activity whereas a decrease in H_2O_2 levels when compared to the only glutamate exposed SH-SY5Y cells. So, it could be speculated that the protective effect of lamotrigine against glutamate toxicity was related to its antioxidant activity. In the study of Kamal et al., it was revealed that the management of epilepsy by lamotrigine could be associated with the possible antioxidant activity, supporting our data [35]. Moreover, in a study evaluating the protective effects of lamotrigine, aripiprazole and escitalopram on depression-induced oxidative stress it was found that lamotrigine had the most protective effect on the oxidative stress within the three drugs [36].

We determined an increase in TNF-α and also in IL-1β, IL-6 levels after induction of 15 mM glutamate in SH-SY5Y cells, and this increase was attenuated by lamotrigine significantly. Similar to our study, in an Alzheimer's model on SH-SY5Y cells it was shown that TNF-α levels increased following treatment with Amyloid-β (Aβ) [37]. Both TNF-α and IL-6 levels elevated in the mechanical trauma injury-induced SH-SY5Y cell model [38]. The levels of inflammatory factors increased in nerve cells under pathological conditions such as ischemia, hypoxemia, mitogens, cytokines and hormones leading to neuronal degeneration [39]. Glial cells including astrocytes and microglia are generally accepted as the major sources of pro-inflammatory cytokine production in the CNS. However, it has been reported that both glial and neuronal cells can produce and release pro-inflammatory cytokines with interaction with chemokines, and adhesion molecules in response to toxic stimuli. Proinflammatory cytokines modulate inflammatory processes [40].

The findings of this study demonstrated a significant reduction of IL-1β, IL-6 and TNF-α levels with lamotrigine in the glutamate-exposed cells. The results of some other studies showed differences in the effects of lamotrigine on cytokine levels. Similar to our findings, it was reported that lamotrigine caused a consistent reduction in IL-6 and TNF-α secretion both in vivo and in vitro lipopolysaccharide (LPS)/concanavalin A (ConA)-induced inflammation model, whereas, only in ConA-induced inflammation model for IL-1β [41,42]. An inhibitory in vitro effect of lamotrigine on TNF-α and IL-1β was seen, whereas, no effect on IL-6 secretion was reported after stimulation of whole blood obtained from healthy female subjects, by toxic shock syndrome toxin-1 [43]. Additionally, IL-1β secretion decreased by lamotrigine with no change on TNF-α and IL-6 levels by stimulation with a combination of anti-CD3 and anti-CD40 antibodies. It was speculated that the variability effects on the proinflammatory cytokines among the studies could be due to the differences in the stimulants, cells and other experimental settings [44].

In conclusion, the results of the present study demonstrate that lamotrigine exerts neuroprotective effects against glutamate

induced toxicity in SH-SY5Y cells by reducing oxidative stress through increased levels of antioxidant enzymes and the proinflammatory cytokines.

Compliance with Ethical Standards

Ethical approval: According to the Institutional Ethical Committee, this study did not require ethics approval as it was conducted on cell lines and the data did not contain patient-specific information.

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Conflict of Interest: The authors declare that they have no conflict of interest.

Author Contributions:

Concept and Design – BTB, EO, NGA, AH; Supervision – BTB, EO, NGA; Resources – BTB, EO, NGA, AH; Materials – BTB, EO, NGA, AH; Data Collection and Processing – BTB, EO, NGA, AH, FT, SA, AK; Analysis and Interpretation – BTB, EO, NGA, AH, FT, SA, AK; Literature Search – BTB, EO, NGA, AH, FT, SA, AK; Writing Manuscript – BTB, EO, NGA, AH, FT, SA, AK; Critical Review – BTB, EO, NGA, AH, FT, SA, AK

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