



## Research article

## Mito-Tempo suppresses autophagic flux via the PI3K/Akt/mTOR signaling pathway in neuroblastoma SH-SY5Y cells

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## HIGHLIGHTS

- Glutamate exposure induces mitochondrial ROS and neurotoxicity in SH-SY5Y cells.
- Increment of MMP loss caused by glutamate-mediated cytotoxicity engaged in the alteration of LC3-I/II and p62 autophagy.
- Mito-Tempo attenuated oxidative stress by increasing superoxide dismutase activity.
- Impaired autophagy also rescued by Mito-Tempo through PI3K/Akt/mTOR signaling pathway.

## ARTICLE INFO

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## ABSTRACT

The generation of excessive mitochondrial reactive oxygen species (mtROS) is associated with glutamate-stimulated neurotoxicity and pathogenesis of Alzheimer's disease (AD). Impaired mitochondrial function is accompanied with oxidative stress that is a significant contributor to initiate autophagy, but the underlying mechanisms are not fully understood. The present study aimed to investigate the neuroprotective effects of Mito-Tempo on glutamate-induced neuroblastoma SH-SY5Y cell toxicity. SH-SY5Y cells were treated with 100  $\mu$ M glutamate in the presence or absence of 50 and 100  $\mu$ M Mito-Tempo for 24 h. Changes in cell viability were measured by MTT assay. Cytotoxicity and intracellular ROS accumulation were also evaluated using lactate dehydrogenase (LDH) activity assay and 2,7-dichlorofluorescein diacetate (DCFDA) Reactive Oxygen Species Assay kit, respectively. Mitochondrial membrane potential was analyzed by tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) staining. Expression of PI3K/AKT/mTOR pathway and autophagy markers, including LC3 (LC3-I/-II) and p62 (SQSTM1) were performed using Western blot analysis. Our results demonstrated that glutamate-exposed cells significantly increased cellular oxidative stress by enhancing ROS production. Glutamate treatment also increased LDH release follows the loss of mitochondrial membrane potential, caused cell viability loss. Treatment with Mito-Tempo not only attenuated the generation of ROS and improved mitochondrial membrane potential but also reduced the neurotoxicity of glutamate in a concentration-dependent manner, which leads to increased cell viability and decreased LDH release. Mito-Tempo has a greater protective effect by enhancing superoxide dismutase (SOD) activity and PI3K/AKT/mTOR phosphorylation. Moreover, Mito-Tempo treatment altered the autophagy process resulting in the decline in the ratio of the autophagy markers LC3-I/-II and p62 (SQSTM1). We propose that Mito-Tempo can improve neuronal properties against glutamate cytotoxicity through its direct free radical scavenging activity and inhibit excessive autophagy signaling pathway, therefore, allow for further studies to investigate the therapeutic potentials of Mito-Tempo in animal disease models and human.

## 1. Introduction

Oxidative stress occurs when there is excessive free radicals production resulting from an insufficiency of the counteracting endogenous antioxidant

defence systems, which plays a crucial role in brain aging and age-related neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD) (Ienco et al., 2011). Since the brain is more vulnerable to free radicals' attack due to its high oxygen consumption and lipid-rich content

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and also low concentrations of antioxidant (Hulbert et al., 2007; Salim, 2017). Therefore, overproduction of free radicals-induced oxidative modification, leading to the generation of reactive oxygen species (ROS) in the mitochondria and mediates apoptosis in neuronal cells, which is induced by a variety of neurotoxic stimuli (Fulda et al., 2010). However, the mechanisms of neuronal cell death in AD remain unclear. Nowadays, neurotoxic agents such as amyloid-beta ( $A\beta$ ) peptides (Mroczko et al., 2018) and metal (Folarin et al., 2017; Garza-Lombó et al., 2018) have been widely used to study the mechanism of neuropathological events in both *in vitro* and *in vivo* models. Glutamate is the major fast excitatory neurotransmitter and is distributed broadly in the mammalian central nervous system (CNS), which plays a pivotal role in brain functions, including synaptic plasticity, memory formation, and learning (Jia et al., 2015). Excessive extracellular glutamate concentration is the key factor that mediates neuronal cell death through N-methyl-D-aspartate (NMDA) receptor activation in a process known as excitotoxicity and subsequent induction of excessive  $Ca^{2+}$  influx, leading to mitochondrial release, which is associated with the apoptotic cell death pathway (Bonfoco et al., 1995). Interestingly, glutamate itself is closely associated with oxidative stress and burst that also participates in autophagy via nicotinic acid adenine dinucleotide phosphate (NAADP)-dependent activation of AMP-activated protein kinase (AMPK), resulting in the release of  $Ca^{2+}$  in neuronal cells, as an early pathological event in AD (Pereira et al., 2017).

The autophagy-lysosomal degradative pathway is essential for maintaining cellular homeostasis and avoid potential cellular stress (Maday et al., 2012). There are three primary types of autophagy in mammalian cells, namely, microautophagy, macroautophagy (also referred to as mitophagy), and chaperone-mediated autophagy (CMA), which is involved in different morphologies and mechanisms and may serve different cellular functions. All three types of autophagy lead to the degradation of cargo to the lysosome and release of the breakdown products back into the cytosol for cell recycling (Parzych and Klionsky, 2014; Kamat et al., 2014). Moreover, neurons have extraordinarily high energy demands, and thereby mitochondrial function is vital for maintaining fundamental neuronal integrity and function (Nicholls and Budd, 2000). In AD, the accumulation of dysfunctional mitochondria in neurons could exacerbate impaired mitophagy (Kerr et al., 2017). Microtubule-associated protein 1A/1B-light chain 3 (LC3) is an indicator of autophagosome formation, leading to autophagy, which acts effectively to scavenged oxidative damage via autophagy-adaptor proteins p62. Additionally, p62 is essential since it is a substrate for LC3 that facilitates selective degradation during autophagy (Liu et al., 2016). Whether insufficient protective autophagy has cytotoxic effects on the accumulation of misfolded protein aggregates can be observed in several neurodegenerative diseases (Matus et al., 2011). Moreover, autophagy has been demonstrated to mediate the clearance of mutant antioxidant enzymes superoxide dismutase (SOD), resulting in a delay in AD progression (Chen et al., 2009). Thus, therapeutic approaches targeting mitochondrial dysfunction and related oxidative stress upon glutamate cytotoxicity may have great promise in the prevention and treatment of AD.

Mito-Tempo is a mitochondria-targeted superoxide dismutase mimetic with effective superoxide scavenging properties, which converts toxic superoxide molecules into hydrogen peroxide or oxygen and subsequently detoxified to oxygen and water by catalase or glutathione peroxidase (Trmka et al., 2009; Liang et al., 2010). Recent studies found that Mito-Tempo has demonstrated beneficial effects on mitochondrial oxidative stress. In mouse models of AD, Mito-Tempo increased SOD activities that could lead to the suppression of superoxide radicals-induced cytotoxicity and improved neuronal survival (Lu et al., 2015; Liang et al., 2009). Moreover,  $A\beta$ -induced mitochondrial dysfunction and neuronal oxidative stress were suppressed by Mito-TEMPO treatment in primary cultured mouse neurons (Hu and Li, 2016). However, the role of mitochondria-targeted antioxidant in AD prevention is still unclear. Based on the antioxidant properties of Mito-Tempo, we therefore hypothesized that Mito-Tempo might exhibit neuroprotective effects against glutamate cytotoxicity. The present study aimed to investigate the therapeutic effects and mechanisms of the mitochondria-targeted antioxidant Mito-Tempo

against glutamate-induced neurotoxicity in SH-SY5Y cells by evaluating cell viability, ROS accumulation, mitochondrial membrane potential, and the expression of PI3K/AKT/mTOR pathway and autophagy markers, LC3-I/-II and p62.

## 2. Materials and methods

### 2.1. Reagents

Glutamate, (2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenyl-phosphonium chloride (Mito-Tempo), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), and anti-actin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-PI3K, anti-p-PI3K, anti-Akt, anti-p-Akt, anti-mTOR, anti-p-mTOR were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-LC3 and anti-p62 (SQSTM1) were supplied by Abcam (Cambridge, CB2 0AX, UK). Cell culture reagents were provided from Thermo Fisher Scientific Inc. (Waltham, MA, USA). All other chemicals used were of analytical reagent grade.

### 2.2. Cell culture and treatments

Human neuroblastoma SH-SY5Y cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA; CRL-2266). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics containing 100 U/ml penicillin and 100 g/ml streptomycin at 37 °C in a humidified 5% CO<sub>2</sub> incubator. Cells were passaged once every 3 days. After five passages, cells were incubated with fresh medium containing glutamate in the presence or absence of Mito-Tempo for 24 h.

### 2.3. Cell viability assay

The cell viability was assessed by the mitochondrial performance, using the MTT reduction assay. To determine whether Mito-Tempo protects against glutamate-induced neurotoxicity, cells were grown in 96-well plates at a density of  $2 \times 10^4$  and treated with different concentrations of glutamate (0–100  $\mu$ M) and Mito-Tempo (0–200  $\mu$ M) in 1% FBS for 24 h. After the incubation period, the medium was removed and replaced by the MTT solution (0.5 mg/ml). Cells were then incubated in an incubator for 2 h. Dimethyl sulfoxide (DMSO) was added to dissolve purple crystals of formazan. The amount of MTT formazan product was measured at 570 nm using a Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments, Inc Winooski, VT, USA). Results are expressed as a percentage of cell survival relative to the respective control from six independent experiments, each in triplicate. Percentages of cell viability are reported as mean  $\pm$  standard error of mean (SEM).

### 2.4. Measurement of lactate dehydrogenase (LDH) release

The release of the cytosolic enzyme LDH into the culture medium is indicative of cellular integrity damage (Rizvi et al., 2016). LDH released was evaluated using the LDH cytotoxicity detection assay kit (Abcam, Cambridge, MA, USA), according to the manufacturer's protocol. Briefly, cells grown in 96-well plates ( $2 \times 10^4$  cells/well) were treated with 100  $\mu$ M glutamate in the presence or absence of 50 and 100  $\mu$ M Mito-Tempo for 24 h. Following 24 h of treatment, the culture medium was transferred into a new 96-well plate and the reaction mixture was added to each well along with the NADH standard, and then incubated at 37 °C for 30 min. The absorbance was measured at 450 nm using a Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments, Inc Winooski, VT, USA). Results are expressed as a percentage of cytotoxicity calculated as the amount of LDH released in response to treatment divided by maximum LDH released. Percentage of LDH releasing cells are expressed as mean  $\pm$  SEM from three independent experiments performed in triplicate.

## 2.5. Determination of intracellular reactive oxygen species (ROS) levels

The intracellular ROS production was evaluated using 2,7-dichlorofluorescein diacetate (DCFDA) Reactive Oxygen Species Assay kit (Thermo Scientific, USA). Briefly, cells grown in 96-well plates ( $2 \times 10^4$  cells/well) were treated as described in section 2.4. Following 24 h of treatment, the medium was completely removed, and cells were incubated with PBS containing 10  $\mu$ M DCFDA in an incubator for 30 min. Subsequently, the 2,7-dichlorofluorescein (DCF) fluorescence was measured using a Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments, Inc Winooski, VT, USA) at 485 nm excitation and 520 nm emission. Results are expressed as a percentage of ROS relative to respective control. Percentage of ROS levels represent mean  $\pm$  SEM of three independent experiments. The experiments were performed in triplicate.

## 2.6. Measurement of superoxide dismutase (SOD) activity

SOD activity was measured using a commercially available SOD assay kit (Cell Biolabs, San Diego, CA, USA), according to the manufacturer's instructions. Briefly, cells grown in 6-well plates ( $3 \times 10^5$  cells/well) were then treated as described in section 2.4. Following 24 h of treatment, cells were harvested and centrifuged at  $2,000 \times g$  for 10 min at 4 °C. Cell pellets were then homogenized in cold HEPES buffer and centrifuged again. The supernatant was collected and the absorbance was read at 450 nm using a Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments, Inc Winooski, VT, USA). The final presentation of SOD activity was units per microliter per microgram of protein. Results are expressed as a percentage of cell survival relative to the respective control from five independent experiments, each in triplicate. Percentage of SOD activity are reported as mean  $\pm$  SEM.

## 2.7. Measurement of mitochondrial membrane potential (MMP)

The MMP was measured using tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) staining, as an indicator of mitochondrial damage. Briefly, cells grown in 96-well plates ( $2 \times 10^4$  cells/well) were treated as described in section 2.4. Following treatment for 24 h, cells were stained with 5  $\mu$ M JC-1 from Thermo Fisher Scientific Inc. (Waltham, MA, USA) for 30 min in an incubator. Then cells were washed twice with PBS and observed under a fluorescence microscope (Nikon, Japan). Five independent experiments were performed in triplicate. Results are expressed as red/green fluorescence intensity ratio and reported as mean  $\pm$  SEM.

## 2.8. Western blot analysis

After treatment, SH-SY5Y cells were harvested and lysed in radioimmunoprecipitation (RIPA) assay buffer with 1% protease inhibitor

cocktail followed by centrifugation at  $2,000 \times g$  for 15 min at 4 °C. The protein concentration was assessed using a bicinchoninic acid (BCA) protein assay kit from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Equal amounts of protein (60  $\mu$ g/lane) were separated on 10–15% SDS-polyacrylamide gel electrophoresis and subsequently transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) at room temperature for 1 h and subsequently probed with primary antibodies at 4 °C overnight. The primary antibodies include the following: anti-PI3K (dilution, 1:1000), anti-p-PI3K (dilution, 1:1000), anti-Akt (dilution, 1:1000), anti-p-Akt (dilution, 1:2000), anti-mTOR (dilution, 1:2000), anti-p-mTOR (dilution, 1:2000), anti-LC3 (dilution, 1:1000), anti-p62 (SQSTM1; dilution, 1:2500), and anti-actin (dilution, 1:5000). The membranes were washed with TBS-T and probed with secondary antibody conjugated with horseradish peroxidase (HRP) at room temperature for 1 h. Protein bands were enhanced using the SuperSignal West Pico chemiluminescence detection kit (Thermo Scientific, USA) and visualized using ChemiDoc XRS + System. Blots were analyzed using Image Lab Software (Bio-Rad, Hercules, CA, USA) and normalized to the expression of actin. A total of six independent experiments, each in triplicates, were assayed and carried out for statistical analysis. Data represent mean  $\pm$  SEM.

## 2.9. Statistical analysis

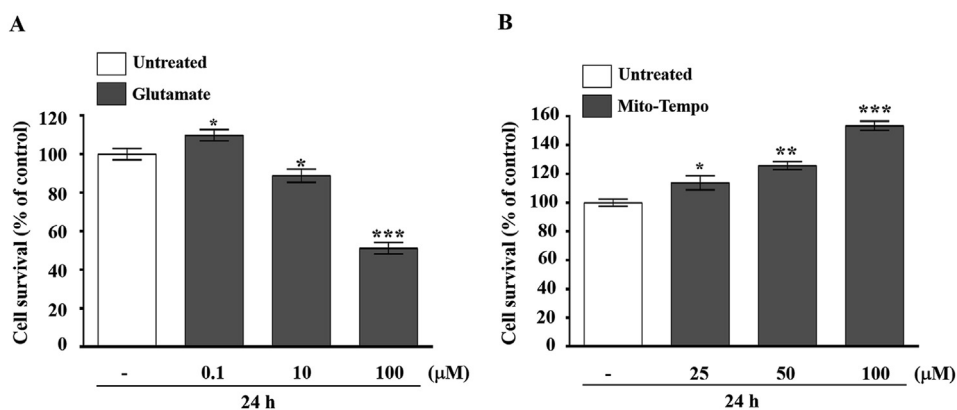
The statistical significance of experimental data was determined by one-way analysis of variance (ANOVA). The pairwise comparisons were combined with the Bonferroni's tests. Differences were considered statistically significant or highly significant at  $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.001$ , respectively. All statistical analyses were conducted using GraphPad Prism 8 for Windows (GraphPad Software, Inc., La Jolla, CA, USA).

## 3. Results

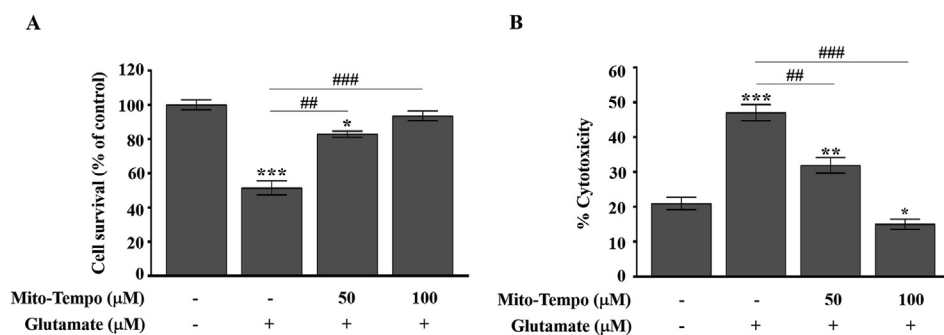
### 3.1. Effect of glutamate and Mito-Tempo on cell viability in SH-SY5Y cells

We initially determine whether treatment with glutamate alone and Mito-Tempo alone was toxic to SH-SY5Y cells using MTT assay. The result showed that treatment with the lowest concentration of glutamate (0.1  $\mu$ M) increased cell proliferation, whereas 10 and 100  $\mu$ M glutamate significantly reduced the number of the living cell to  $88.86 \pm 3.45\%$  and  $51.12 \pm 2.97\%$ , respectively, as compared to the untreated cells (Figure 1A), possibly due to excitotoxic effects of glutamate.

On the other hand, Mito-Tempo-treated groups showed no cytotoxicity effect for cells. A significant increase in cell viability was also detected following treatment with Mito-Tempo at 25  $\mu$ M ( $113.85 \pm 4.93\%$ ), 50  $\mu$ M ( $125.86 \pm 2.85\%$ ), and 100  $\mu$ M ( $153.45 \pm 3.16\%$ ),



**Figure 1.** Concentration-response effect of glutamate and Mito-Tempo on cell viability in SH-SY5Y cells by MTT assay. (A) The effect on cell viability following treatment with various concentrations of glutamate and (B) Mito-Tempo for 24 h. Data are presented as mean  $\pm$  SEM from at least three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  vs. untreated group.



**Figure 2.** Effect of Mito-Tempo against glutamate-induced cytotoxicity in SH-SY5Y cells. SH-SY5Y cells were treated with 100 μM glutamate in the presence or absence of 50 and 100 μM Mito-Tempo for 24 h. (A) Cell viability was assessed by MTT assay. (B) LDH release was determined using a cytotoxicity detection kit. Data are presented as mean ± SEM from at least three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  vs. untreated group. ## $p < 0.01$  and ### $p < 0.001$  vs. glutamate alone group.

respectively (Figure 1B). However, using an MTT assay, we determined that 50 and 100 μM Mito-Tempo were the optimal concentrations that did not adversely affect SH-SY5Y cell viability. It will be used in all subsequent experiments.

### 3.2. Mito-Tempo protected SH-SY5Y cells against glutamate-induced cytotoxicity

To evaluate the protective efficacy of Mito-Tempo against glutamate-induced cellular damage, SH-SY5Y cells were treated with 100 μM glutamate in the presence or absence of 50 and 100 μM Mito-Tempo for 24 h. The result showed that 50 and 100 μM Mito-Tempo significantly restored cell viability to  $82.90 \pm 1.78\%$  and  $93.56 \pm 2.85\%$ , respectively, indicating that Mito-Tempo could protect the cells from glutamate toxicity (Figure 2A). To further confirm the cell membrane permeability, LDH was measured in the culture medium. The increase in LDH release in the culture medium was 2.60-fold greater in the glutamate treated cells when compared to that in the untreated cells, indicating a compromise in cell membrane integrity. However, Mito-Tempo at a concentration of 50 and 100 μM significantly reduced glutamate-induced LDH release in a concentration-dependent manner down to 1.51-fold and 0.32-fold, respectively, as compared to the glutamate-treated cells, suggesting that Mito-Tempo may block or alleviate cellular injury in SH-SY5Y cells.

### 3.3. Mito-Tempo inhibited glutamate-induced intracellular accumulation of ROS

Excessive ROS levels were identified as a major reason for glutamate-dependent cell death (Ramalingam and Kim, 2012). In our study, the accumulation of ROS was evaluated by intracellular ROS assay kit. Incubation with glutamate for 24 h could catalyze the oxidation of DCFDA to its fluorescent product DCF resulted in intracellular ROS levels drastically increased to  $(234.45 \pm 6.15\%)$  compared with untreated cells ( $100 \pm 7.10\%$ ) (Figure 3A). However, glutamate-induced intracellular ROS level was remarkably mitigated by Mito-Tempo, which significantly

decreased in a concentration-dependent manner at 50 ( $103.78 \pm 6.67\%$ ) and 100 μM ( $78.12 \pm 5.67\%$ ), respectively (Figure 3A). The result indicated that Mito-Tempo may help in maintaining intracellular redox balance by inhibiting the mitochondrial oxidant accumulation in glutamate-rich environments.

### 3.4. Effect of Mito-Tempo on anti-oxidative enzymes activity challenged by glutamate

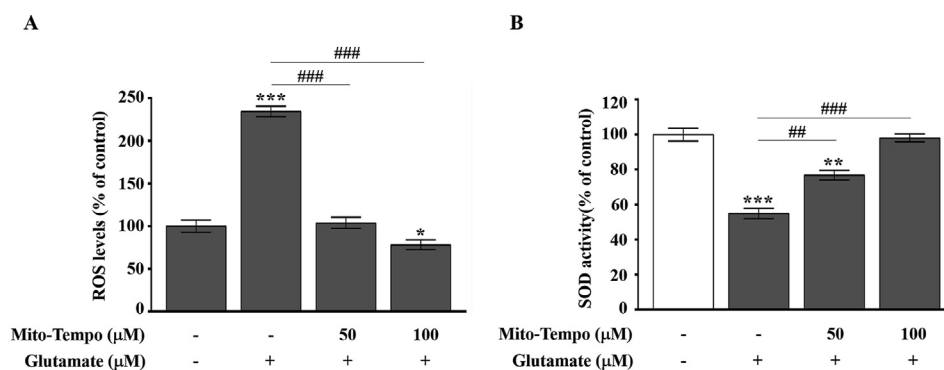
To determine whether Mito-Tempo regulates the expression of SOD antioxidant enzymes against oxidative stress. Following exposure to glutamate for 24 h, SOD activity was significantly lower in the glutamate-exposures group ( $54.95 \pm 2.95\%$ ) than that in the untreated group, whereas treatment of cells with 50 and 100 μM Mito-Tempo restored SOD activity to  $76.78 \pm 2.77\%$  and  $98.12 \pm 2.27\%$ , respectively (Figure 3B).

### 3.5. Mito-Tempo inhibited glutamate-induced mitochondrial dysfunction

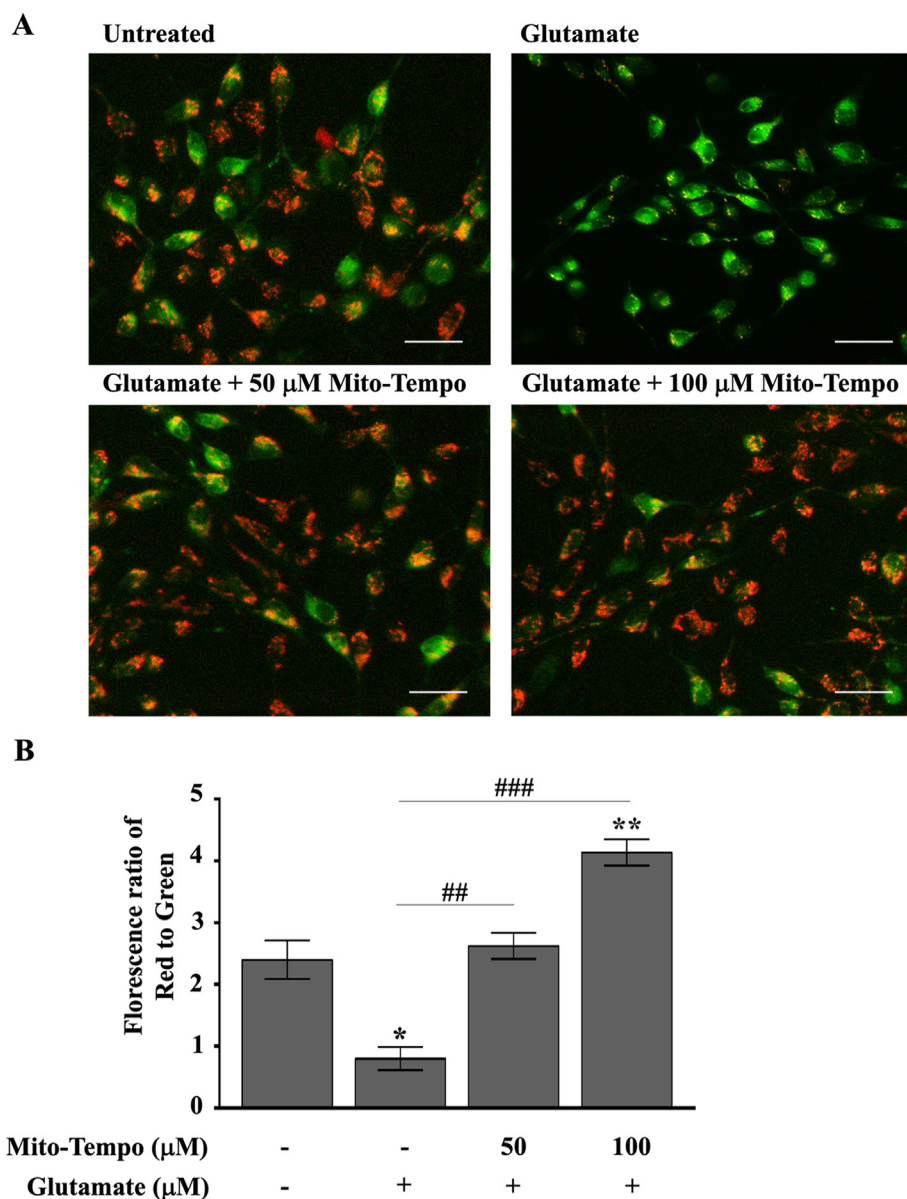
Glutamate-induced oxidative damage is critically involved in a cellular injury, including mitochondrial dysfunction (Savolainen et al., 1995). Therefore, we hypothesized that mitochondria-targeted antioxidant, Mito-Tempo may enhance mitochondrial membrane potential (MMP) from glutamate-induced cellular injury. The MMP was measured using JC-1 fluorescent dyes that are capable of gathering in the matrix of mitochondria and forms aggregate, which can leak out to the cytosol as a monomer if the MMP loss and produce green fluorescence. Compared with the untreated cells, glutamate markedly decreased MMP, while Mito-Tempo efficiently restored MMP (Figure 4A-B).

### 3.6. Mito-Tempo protected against glutamate-induced cytotoxicity via the PI3K/AKT/mTOR pathway

The PI3K/AKT/mTOR signaling pathway plays a role in activation of autophagy and neuronal survival (Hernández-Damián and Pedraza-Chaverri, 2014). We further investigated the possible mechanism



**Figure 3.** Effect of Mito-Tempo on antioxidant properties in SH-SY5Y cells. (A) Free radical production levels, as determined using the fluorescent probe DCF, and (B) the activity of the SOD antioxidant enzymes after treated with 100 μM glutamate in the presence or absence of 50 and 100 μM Mito-Tempo for 24 h. Data are presented as mean ± SEM from at least three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  vs. untreated group. ## $p < 0.01$  and ### $p < 0.001$  vs. glutamate alone group.



**Figure 4.** Inhibitory effect of Mito-Tempo on glutamate-induced mitochondrial dysfunction in SH-SY5Y cells. (A) JC-1 staining in SH-SY5Y cells, followed exposure to 100  $\mu\text{M}$  glutamate in the presence or absence of 50 and 100  $\mu\text{M}$  Mito-Tempo for 24 h, scale bar = 100  $\mu\text{m}$  under 200 $\times$  magnification. JC-1 fluorescent dyes can gather in the matrix of mitochondria and produce red fluorescence. As the mitochondrial membrane potential loss, JC-1 can not gather the matrix which leaks out to the cytosol as a monomer and then produce green fluorescence. (B) The ratio of the red/green fluorescence optical density. Data are presented as mean  $\pm$  SEM from at least three independent experiments. \* $p < 0.05$  and \*\* $p < 0.01$  vs. untreated group. ## $p < 0.01$  and ### $p < 0.001$  vs. glutamate alone group.

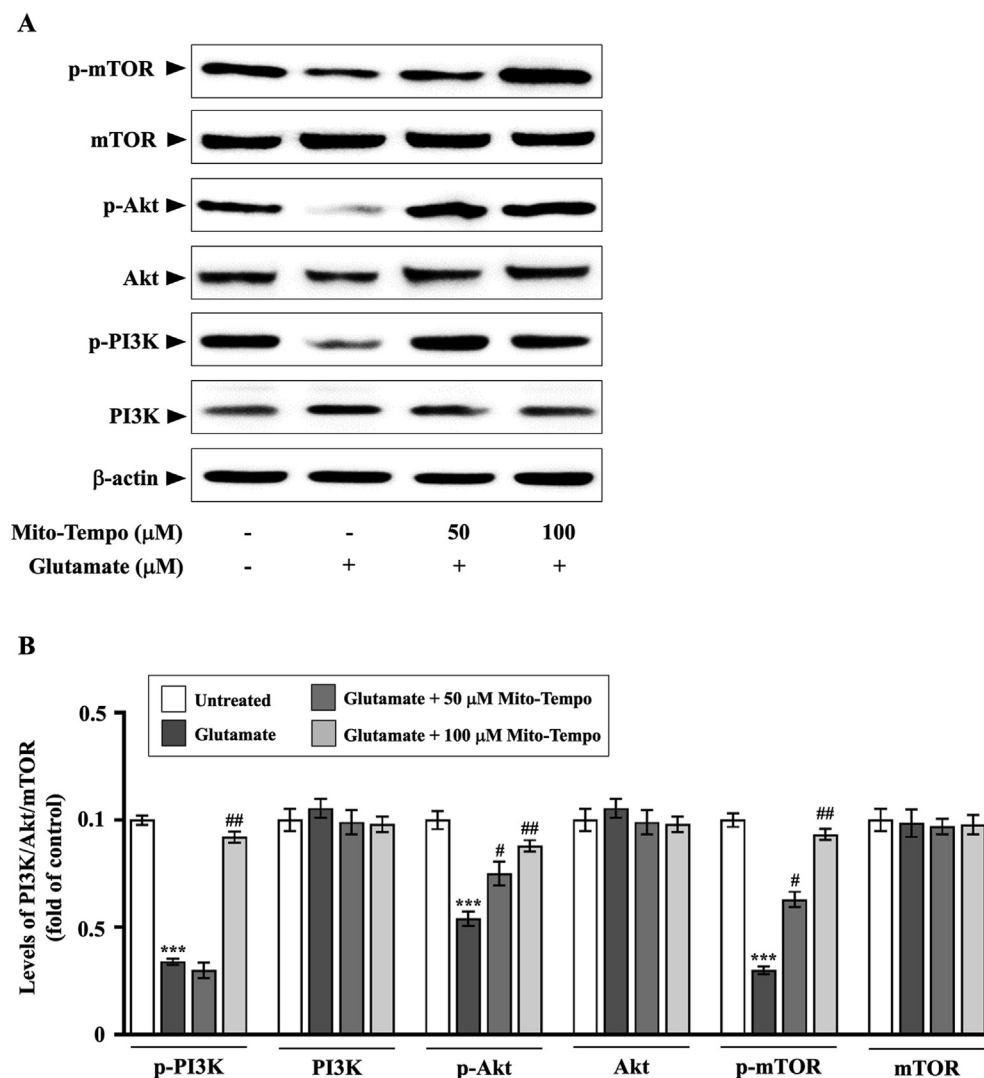
of Mito-Tempo on autophagy during glutamate-induced cytotoxicity through the PI3K/Akt/mTOR signaling pathway using Western blot analysis. The expression levels of p-PI3K, p-Akt, p-mTOR significantly decreased in the glutamate-treated cells compared with the untreated cells (Figure 5A-B).

### 3.7. Mito-Tempo suppressed glutamate-induced autophagy flux

The loss of MMP is the crucial initiating signal to trigger the process of autophagy in neural cells (Pereira et al., 2017). Next, we decided to assess whether Mito-Tempo may prevent autophagy flux mediated by glutamate cytotoxicity in SH-SY5Y cells. Therefore, protein levels of LC3-I/II, a marker for autophagy activation, and p62 (SQSTM1), a marker for autophagic/lysosomal degradation were determined using Western blot analysis. Immunoblots showed that the conversion of LC3-I to LC3-II gradually increased, while levels of p62 (SQSTM1) substantially decreased after treated with glutamate alone, indicated that glutamate increased autophagy flux in SH-SY5Y cells. In contrast, Mito-Tempo prevented the conversion of LC-I to LC3-II and an increase in p62 (SQSTM1) levels (Figure 6A-C).

## 4. Discussion

Oxidative stress in neuronal cells promotes the excessive amounts of ROS that trigger downstream events of the apoptosis pathway, and leads to mitochondrial injury (Liu et al., 2017). Many pieces of evidence reported that excessive glutamate concentration causes neuronal cell death, namely excitotoxicity via mitochondria dysfunction, which is associated with the pathological process underlying AD (Wang and Reddy, 2017). Recently, mitochondrial-targeted antioxidant, NMDA receptor antagonist, could delay glutamate-mediated mitochondrial dysfunction and cellular injury, which is related to oxidative stress in the etiology of AD (Wang and Reddy, 2017). It suggested its potential therapeutic approaches targeting mitochondrial dysfunction directly at the major site of cellular ROS production, where they certainly scavenge ROS. The present study demonstrated that Mito-Tempo, mitochondria-targeted antioxidant, may exert its neuroprotective activities through free radical scavenging, stabilization of mitochondrial membrane potential, and prevention of autophagy activation against glutamate-induced cytotoxicity.



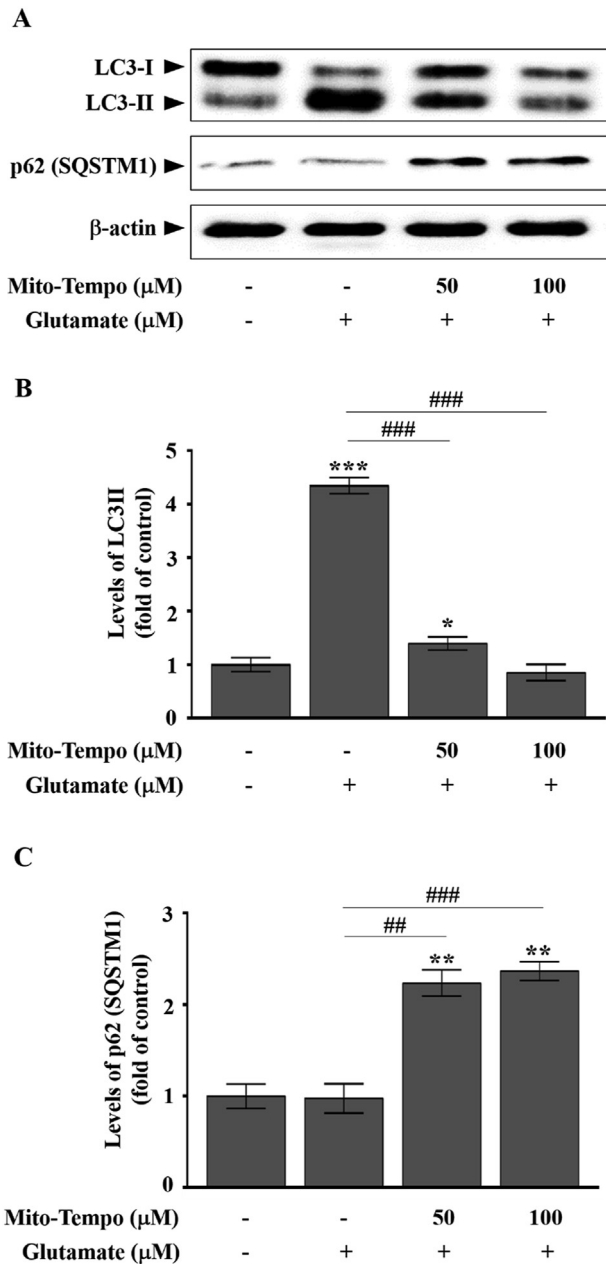
**Figure 5.** Effect of Mito-Tempo protected against glutamate toxicity via the PI3K/Akt/mTOR pathway in SH-SY5Y cells. (A) Western blot was performed to detect the protein expression levels of the PI3K/Akt/mTOR signaling pathways in SH-SY5Y cells, followed by treatment with 50 and 100  $\mu$ M of Mito-Tempo under a glutamate-rich condition for 24 h. (B) The expression levels of the PI3K/Akt/mTOR signaling pathways were normalized against  $\beta$ -actin and represented as the fold-increase relative to that of the untreated cells. Data are presented as mean  $\pm$  SEM from at least three independent experiments. \*\*\* $p$  < 0.001 vs. untreated group. # $p$  < 0.05 and ## $p$  < 0.01 vs. glutamate alone group. The uncropped versions of figures are presented in Fig. S5A.

Administration of the lowest concentration of glutamate (0.1  $\mu$ M) increased cell viability suggesting that glutamate, a major neurotransmitter that serves as a fuel for ATP synthesis, neuronal transmission, and cell communication, could enhance the intracellular ATP content and energy state of the cell (Stamoula et al., 2015; Piccirillo et al., 2018). Remarkably, we found that 10 and 100  $\mu$ M glutamate could induce cellular injury in SH-SY5Y cells as indicated by decreased cell viability using MTT assay. Since MTT assay is used as an indirect measure of cell death which actually measures the cellular metabolic activity of mitochondrial enzymes (Cumings and Schnellmann, 2004), its reduced signal could reflect any manner of cell death, such as apoptosis and necrosis (Martínez et al., 2020). Although we did not observe the cell death assay, the LDH release was performed to represent an early indicator for the apoptotic cell death. Increasing LDH release induced by glutamate, providing direct evidence of neurotoxicity that can be implied to neuronal cell death (Lobner, 2000; Zhang and Bhavnani, 2005). It strongly resembles with those of previous *in vitro* studies (Noh et al., 2006; Kang et al., 2007; Hu et al., 2012). However, this neurotoxic action was decreased when treated with an optimal concentration of Mito-Tempo that did not adversely affect cell viability for 24 h, indicated that Mito-Tempo could directly prevent glutamate-induced cell injury by enhancing ROS-clearance and restoration of mitochondrial membrane potential (Trnka et al., 2009; Zhang et al., 2017) and due to its anti-apoptotic effects (Hu et al., 2012).

Under physiological conditions, ROS are produced by the respiratory chain during oxidative phosphorylation and maintained in a low

concentration. In contrast, impairment of them is the primary source of free radicals, including superoxide ( $O_2^-$ ), hydroxyl radicals ( $OH^\cdot$ ), and hydrogen peroxide ( $H_2O_2$ ) in most cell types (Bak and Weerapana, 2015). Many studies have shown that glutamate-induced neurotoxicity enhanced  $H_2O_2$  levels caused by oxidative damage (Terzioğlu Bebitoğlu et al., 2020). Similarly, our study also demonstrated that glutamate could elevate levels of intracellular ROS in SH-SY5Y cells, whereas Mito-Tempo inhibited mitochondrial ROS production, suggesting that Mito-Tempo acts on SH-SY5Y cells not only eliminating ROS but also through inhibiting the redox status of the mitochondria. Since increased ROS burden that incurs DNA damage and lipid oxidation, thereby lead to decrease mitochondrial membrane potential, eventually promote the apoptosis pathway (Wang et al., 2014; Angelova and Abramov, 2018).

The major antioxidant enzymes, basically including SOD, catalase (CAT), and glutathione peroxidase (GPX) are directly involved in the neutralization of free radicals. SOD is the first line of defence against free radicals that catalyzes the dismutation of superoxide anion radical ( $O_2^{\cdot-}$ ) into  $H_2O_2$ , which is then converted into  $H_2O$  and  $O_2$  by CAT or GPx (Zhan et al., 2004; Ighodaro and Akinloye, 2018). It was reported that SOD activity in the brain is significantly higher than CAT activity. Accordingly, we proposed that the protective effect of mitochondria-targeted antioxidant on glutamate toxicity is due most likely to its antioxidant properties of SOD in SH-SY5Y cells. The result showed that Mito-TEMPO restored the SOD level against glutamate-induced cellular injury. It confirms that Mito-Tempo enhanced antioxidant SOD activity due to its superoxide radical



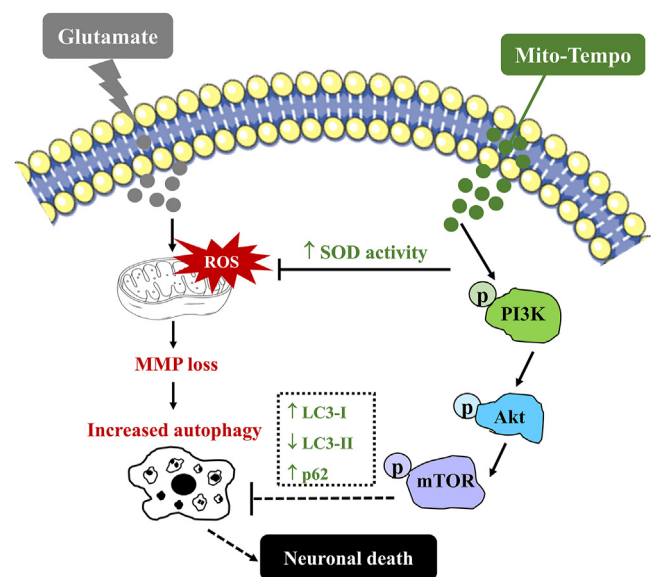
**Figure 6.** Effect of Mito-Tempo on autophagy flux induced by glutamate cytotoxicity in SH-SY5Y cells. (A) Western blot analysis illustrating the expression levels of LC3-I/-II and p62 (SQSTM1), followed by treatment with 50 and 100  $\mu\text{M}$  of Mito-Tempo under a glutamate-rich condition for 24 h. (B) The expression levels of LC3-I/-II and (C) p62 (SQSTM1) were normalized against  $\beta$ -actin and represented as the fold-increase relative to that of the untreated cells. Data are presented as mean  $\pm$  SEM from at least three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  vs. untreated group; ## $p < 0.01$  and ### $p < 0.001$  vs. glutamate alone group. The uncropped versions of figures are presented in Fig. S6A.

scavenging properties that enhance the antioxidant defense mechanism in intact cells, as reported previously (Dikalova et al., 2010).

In neuronal cultures, apoptotic cell death can involve assays for determining loss of plasma membrane integrity or measurements of mitochondrial injury, which is an early indicator in the apoptotic mechanism (Shearman et al., 1994; Koh et al., 1995; Lobner, 2000). Moreover, changing in MMP precedes a mitochondrial dysfunction is a primary event in glutamate-induced neurotoxicity, resulting in cellular

bioenergetic collapse and increased levels of ROS, which may cause additional damage to cellular function that is reported in neurodegenerative diseases (Bak et al., 2016; Zhou et al., 2018; Schinder et al., 1996). Herein, we observed abnormal ROS levels upon glutamate treatment accompanied by MMP reduction, implying loss of mitochondrial integrity in SH-SY5Y cells. In the present study, mitochondrial dysfunction is important preceding events promoted by glutamate-induced neuronal death. Using Mito-Tempo, we found that mitochondrial function was improved by rescuing MMP from glutamate-induced excitotoxicity, suggesting that Mito-Tempo may preserve mitochondrial integrity that could prevent cell apoptosis by blocking the formation of ROS-dependent cell death. Current evidence suggests that Mito-Tempo prevents  $\text{Ca}^{2+}$  overload and attenuate mitochondrial permeability transition pore (MPTP) opening associated with the suppression of the JNK-c-Jun cell death signaling cascade (Liang et al., 2010).

Autophagy plays a vital role in both physiological functions and situations of cell stress in order to maintain proper neuronal function and homeostasis by eliminating damaged organelles. Autophagy is also induced by the accumulation of ROS levels under a variety of stressors. Hung et al. (2009) found that the autophagic process is involved in A $\beta$ -induced neurotoxicity. In contrast, defects in autophagic regulation may impair the clearance of A $\beta$  and enhance neuronal cell death (Hung et al., 2009). Excessive autophagy is an important cause for cell death, and particularly mitophagy participates in mitochondrial dysfunction (Parzych and Klionsky, 2014; Kamat et al., 2014). However, the role of autophagy is complicated and controversial. Nevertheless, autophagy is widely studied in neurodegenerative diseases and chemical-induced neurotoxicity. Autophagy is well-known as important regulators of cell growth and survival (Jafari et al., 2019; Xu et al., 2020), which can be modulated by multiple signaling pathways, including the PI3K/Akt/mTOR pathway. Previous study demonstrated that excessive autophagy was inhibited via activating the Akt pathway in a neuroinflammatory rat



**Figure 7.** Schematic diagram of the possible mechanism of Mito-Tempo against glutamate-induced cytotoxicity in SH-SY5Y cells. Extracellular glutamate concentration causes redox imbalance, which subsequently decreased MMP and increased autophagy signaling pathway (LC3-I/LC3II and p62), leading to initiate neuronal death. Mito-Tempo may counteract glutamate-induced neurotoxicity by increased SOD activity, which might contribute to inhibit ROS production. Mito-Tempo may provide neuronal protective effects against glutamate insult via modulating the function of the PI3K/Akt/mTOR pathway, which is essential pathway in promoting cell survival. Taken together, these events protect SH-SY5Y cells from oxidative stress and promote SH-SY5Y cells survival.

model (Justin-Thenmozhi et al., 2018). In addition, activation of PI3K downstream can phosphorylate mTOR, which is a key regulator of autophagy (Xu et al., 2020). Consistently, our results demonstrated that Mito-Tempo significantly increased the expression levels of p-PI3K, p-Akt, and p-mTOR, indicating that Mito-Tempo may associate in autophagy function through activating the PI3K/Akt/mTOR pathway. Interestingly, we found that glutamate could increase autophagic flux by decrease p62 (SQSTM1) expression and enhance the conversion of LC3-I to LC3-II, resulting in autophagic cell death, whereas Mito-Tempo decreased the expression of LC3-II and upregulated the expression of p62. The p62 is an autophagy-adaptor protein that binds directly to LC3 to facilitate the degradation of ubiquitinated protein aggregates by autophagy, which brings about therapeutic interventions to inhibit excessive autophagy or knockdown of endogenous p62 to alleviate disease progression in AD (Su et al., 2018; Towers and Thorburn, 2016; Nihira et al., 2014). These findings also support the neuroprotective activities of Mito-Tempo on the regulation of autophagy upon free radicals and loss of mitochondrial membrane potential conditions. Taken together, these results show that Mito-Tempo may be a promising protective strategy for neurological pathologies such as Alzheimer's and Parkinson's diseases.

## 5. Conclusion

In conclusion, our findings may yield more insight into the mechanism of Mito-Tempo against glutamate-induced neurotoxicity in neuroblastoma SH-SY5Y cells. Mito-Tempo can partially attenuate glutamate-mediated cytotoxicity and impairment of autophagy in SH-SY5Y cells upon the enhancement of oxidative stress (Figure 7). These results also encourage further study into the *in vivo* activity of Mito-Tempo and its possibility of clinical application in AD and other neurodegenerative diseases.

## Declarations

### Author contribution statement

Sirirak Mukem: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed to reagents, materials, analysis tools or data; Wrote the paper.

Tipsuda Thongbuakaew: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Kanjana Khornchatri: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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### Data availability statement

Data will be made available on request.

### Declaration of interests statement

The authors declare no conflict of interest.

### Additional information

Supplementary content related to this article has been published online at <https://doi.org/10.1016/j.heliyon.2021.e07310>.

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