

Research Article

Protection of SH-SY5Y Neuronal Cells from Glutamate-Induced Apoptosis by 3,6'-Disinapoyl Sucrose, a Bioactive Compound Isolated from Radix Polygala

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The neuroprotective effects of 3,6'-disinapoyl sucrose (DISS) from Radix Polygala against glutamate-induced SH-SY5Y neuronal cells injury were evaluated in the present study. SH-SY5Y neuronal cells were pretreated with glutamate (8 mM) for 30 min followed by cotreatment with DISS for 12 h. Cell viability was determined by (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and apoptosis was confirmed by cell morphology and flow cytometry assay, evaluated with propidium iodide dye. Treatment with DISS (0.6, 6, and 60 $\mu\text{mol/L}$) increased cell viability dose dependently, inhibited LDH release, and attenuated apoptosis. The mechanisms by which DISS protected neuron cells from glutamate-induced excitotoxicity included the downregulation of proapoptotic gene Bax and the upregulation of antiapoptotic gene Bcl-2. The present findings indicated that DISS exerts neuroprotective effects against glutamate toxicity, which might be of importance and contribute to its clinical efficacy for the treatment of neurodegenerative diseases.

1. Introduction

Although neurological insults are diverse in nature, there are common mechanisms of cell injury, and glutamate toxicity plays an integral role in a variety of neurobiology of disorders such as Parkinson's disease, Alzheimer's disease, epilepsy ischemic stroke, anxiety, and depression [1, 2]. Glutamate is the major fast excitatory neurotransmitter in the mammalian central nerve system. Thus far, a distinct glutamate-induced cell death pathway has been identified. The excitotoxic pathway relies on hyperactivation of glutamate receptors [3]. Besides, it has been proposed that the combination of antidepressant drugs that elevate the noradrenergic neurotransmission with drugs that modify the glutamatergic system could be an option in treating depression [4].

3,6'-disinapoyl sucrose (DISS, Figure 1(a)) is the active oligosaccharide ester component found in the root of

Polygala tenuifolia Willd (Radix Polygala). Recorded as "YuanZhi" in the Pharmacopoeia of the People's Republic of China, the root has been used in traditional medicine as, among other things, an expectorant, tonic, tranquilizer, and antipsychotic agent. Results from previous studies indicated that DISS had notable antidepressant effects in pharmacological depression models, an action closely related to the potentiation of central 5-hydroxytryptamine (5-HT) and norepinephrine (NE) systems [5]. Our recent study also found that DISS increased expression in the hippocampus of three noradrenergic-regulated plasticity genes (laminin, CAM-L1, and CREB) and one neurotrophic factor (BDNF) [6]. Based on our results, we hypothesized that DISS might have neuroprotective property. Thus, the authors used the human neuroblastoma SH-SY5Y cell line, with a low degree of differentiation and metabotropic glutamate receptors [7], utilized glutamate as an insult to induce SH-SY5Y neuronal

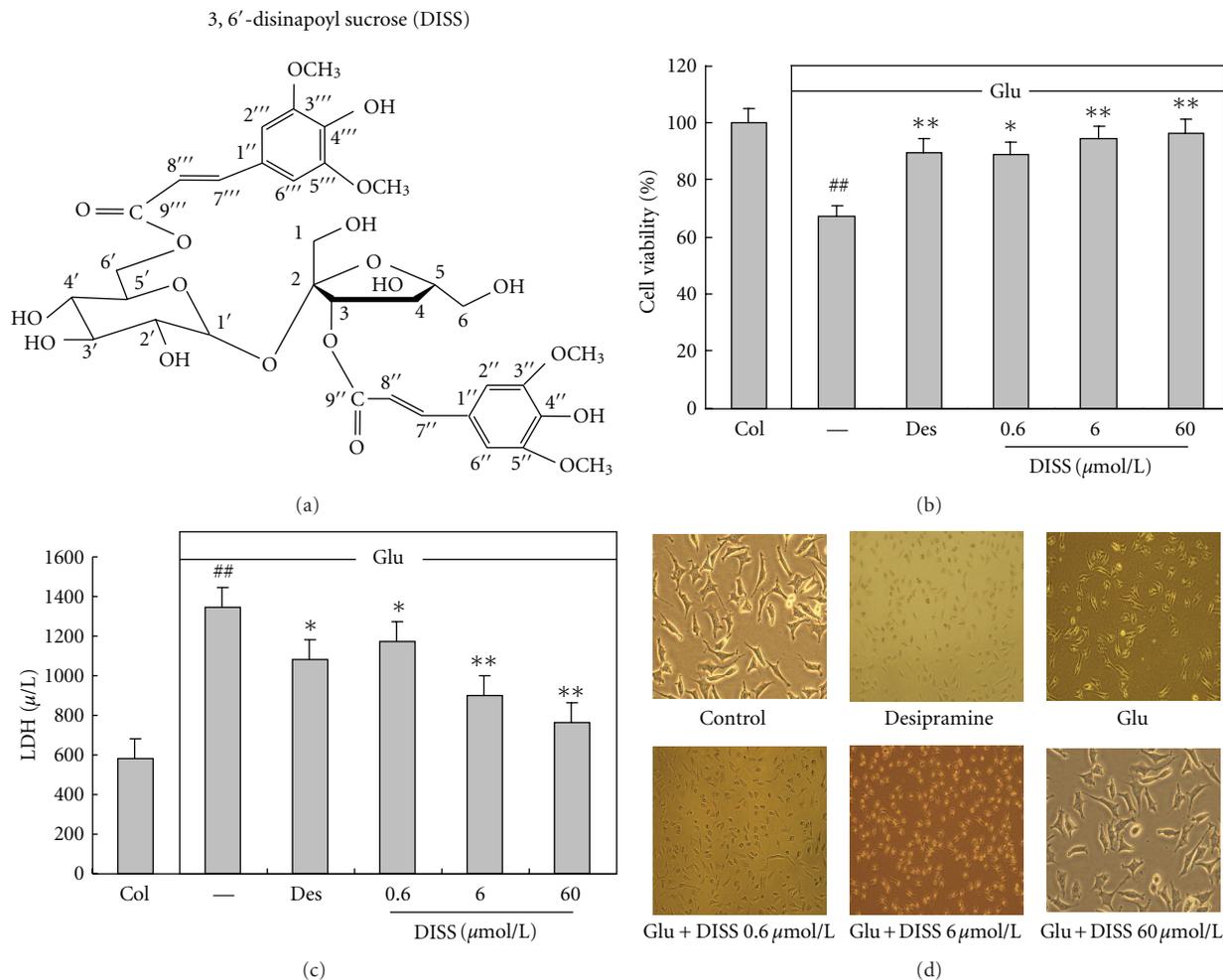


FIGURE 1: Protective effects of DISS on glutamate- (Glu-) induced cytotoxicity in SH-SY5Y cells. (a) Chemical structure of 3,6'-disinapoyl sucrose (DISS). (b) Effects of DISS treatment on SH-SY5Y cells viability decrease induced by glutamate (8 mM) exposure. (c) Effects of DISS treatment on lactate dehydrogenase (LDH) release of SH-SY5Y cell damaged by glutamate. (d) Effect of DISS on glutamate- (Glu-)induced morphological alterations in SH-SY5Y cells. All data were expressed as mean \pm S.D. of three experiments. ^{##} $P < 0.01$, compared with normal cultures; ^{*} $P < 0.05$, ^{**} $P < 0.01$ compared with Glu alone group. Co: Control; Des: desipramine, which is positive drug.

cell injury, and aimed to investigate the neuroprotective effects of DISS on glutamate-induced apoptosis as well as its mechanisms.

2. Resource and Preparation of DISS

DISS, used in present study, with a purity of over 90%, was extracted from the roots of *P. tenuifolia*, which were purchased from Traditional Chinese Medicinal (TCM) pharmacy, Chinese People's Liberation Army (PLA) General Hospital (Beijing, China); a voucher specimen (NU-80617) was deposited in the Herbarium there. Three-month air-dried roots (965.27 g) were extracted with 60% EtOH (8:1) at room temperature for 2 weeks. The dry extract obtained was then subjected to open column chromatography (CC) packed with macroporous resin (1300 Version). The 50% aqueous-ethanol fraction was concentrated under reduced pressure using a rotary evaporator and lyophilized into

powders, further chromatographed on the silica gel column, and eluted by CHCl_3 -MeOH-H₂O to get DISS [8]. The structures were identified by a combination of spectral methods (UV, IR, MS, and NMR), with purity of over 90%.

2.1. Cell Culture and Treatment. The human neuroblastoma SH-SY5Y was provided by Department of Obstetrics and Gynecology in Chinese PLA General Hospital. The human neuroblastoma SH-SY5Y cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 U/mL streptomycin in a humid atmosphere of 5% CO₂ and 95% air at 37°C. SH-SY5Y cells were plated in plates. Confluent SH-SY5Y cells were washed twice with D-Hanks solution before the addition of 0.25% trypsin-EDTA. The flask was left for 2-3 min at room temperature (close to 20°C), after which the cells were detached, resuspended in full medium, counted, and seeded into 96-well plates at a density of

1×10^4 cells/well in normal growth medium. After 24 h, the cells were completely attached to the well bottom. Then cells were exposed to L-Glutamate (Glu) for 0.5 h, followed by treatment with various concentrations of DISSs (0.6, 6, and 60 $\mu\text{mol/L}$) and desipramine as a positive drug. DISS and glutamate were dissolved in dimethyl sulfoxide (DMSO) before added in cell. Final drug concentrations were obtained by dilution of stock solutions in experimental media. Final concentrations of DMSO were always less than 0.01%, which was proved to have no effects on cell viability. The absorbance was read at 570 nm with DMSO as the blank. The DISS dose range was chosen from previous results on preliminary experiments. Cell viability and LDH activity were measured after 12 h incubation at 37°C.

2.2. Cell Viability. Cell viability was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [9]. Briefly, after 0.5 h exposure to Glu, 20 μL of MTT (2 mg/mL in PBS) were added to each well and the cells were incubated at 37°C for 4 h. The supernatants were aspirated carefully, 150 μL of DMSO were added to each well to dissolve the precipitate, and absorbance was measured at 570 nm using a microplate reader (Spectra MR, Dynex, USA). Cell death was determined by measuring the lactate dehydrogenase (LDH) activity using commercially available kits from the Nanjing Jiancheng Bio-company (Nanjing, China).

2.3. Flow Cytometric Detection of Apoptotic Cells. After treatment, 5×10^6 cells were trypsinized, washed in PBS, and centrifuged at 1000 g for 5 min. Then cells were fixed in 70% ethanol overnight. The pellet was rinsed twice and resuspended in 0.5 mL PBS (containing 50 $\mu\text{g/mL}$ RNaseA), incubated for 30 min at 37°C. PI (50 $\mu\text{g/mL}$) was added, mixed gently, and incubated for 30 min at 4°C in dark. The samples were then read in a Becton Dickinson flow cytometer (USA) at 488 nm excitation. A 600 nm bandpass filter for PI detection was used. Ten thousand cells in each sample were analyzed, and the percentage of apoptotic cells accumulating in the sub-G1 peak was calculated by CellQuest software.

2.4. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from cells cultured in the 25 cm² plastic flasks with 5×10^6 cells using Trizol reagent (Gibco BRL) as described by the manufacturer. And its reverse transcription to cDNA was performed using iScript cDNA Synthesis Kit (Bio-Rad, Calif, USA), according to the manufacturer's protocol. Human β -actin, bcl-2, and Bax primers were synthesized by BM (Biomed, China) according to the following sequences [10]. β -actin: forward 5'-GGACATCCGCAAAGACCTGTA-3', reverse 5'-ACATCT-GCTGGAAGGTGGACA-3'; bcl-2: forward 5'-TTTGAG-TTCGGTGGGGTTCATC-3', reverse 5'-CCAGGAGA-AAT-CAAACAGAGG-3'; bax: forward 5'-TTTGCTTCAGGG-TTTCATCC-3', reverse 5'-GCCACTCGGAAAAAGACC-TC-3'. SsoFast EvaGreen Supermix (Bio-Rad, Calif, USA) was used for real-time PCR to detect abundance of PCR products among samples. Standard curves were generated

for each gene, and transcript values were calculated relative to dilution series of cDNA as described in Bio-Rad iQ5 System (Calif, USA). Target quantities were normalized to 18S ribosomal RNA, calibrated using control values, and defined as a value of "1.0". All quantities were expressed as n-fold relative to the calibrator (control).

2.5. Statistical Analysis. Data are presented as the mean \pm S.D. Statistical comparisons were made by one-way ANOVA followed by Tukey's post hoc test. Values of $P < 0.05$ and $P < 0.01$ were considered significant.

3. Result

3.1. Effect of DISS on Cell Viability and LDH Release. As shown in Figures 1(b) and 1(c), 8 mM of Glu was used to induce SH-SY5Y cell injury, whose group was only $67.28 \pm 1.2\%$ viable cells and increased the LDH level as compared to control cells, while treating the cells with DISS at different concentrations (0.6, 6, and 60 $\mu\text{mol/L}$) increased the viability of cells and inhibited LDH release, both with dose-dependent. Moreover the effects of DISS could also be confirmed by the morphological observation (Figure 1(d)). There was a significant injury in SH-SY5Y cells after treatment with Glu, including the disappearance of cellular processes, decrease of the refraction, and falling to pieces. The damage in groups of DISS-treated cells was greatly decreased.

3.2. Flow Cytometry Assay. The nuclear staining assay was used to evaluate the morphological changes of apoptosis in SH-SY5Y cells. As shown in Figure 2, control cells without the treatment with Glu exhibited uniformly dispersed chromatin and intact cell membrane. The cells, treated with 8 mM Glu, increased the percentage of apoptotic cells from 0.96% to 4.93%, compared to control cells. However, in 0.6, 6, and 60 $\mu\text{mol/L}$ DISS-treated cells, cell apoptosis induced by Glu was markedly decreased to 2.58%, 2.36%, and 1.95%, respectively.

3.3. Effect of DISS on the Expression of Bcl-2 and Bax and in Glu-Induced Cells. The effects of DISS on the expression of the apoptotic genes Bax and Bcl-2 were also examined in Glu-injured SH-SY5Y cells. As shown in Table 1, Glu enhanced 2-fold expression of Bax increased, on the contrary, it decreased the levels of Bcl-2 comparison with the normal control group. However, DISS (60 $\mu\text{mol/L}$) treatment inhibited the increase of Bax and the decrease of Bcl-2 dramatically at 12 h of glutamate exposure.

4. Discussion

Glutamate, a major excitatory amino acid neurotransmitter in central nervous system, mediates several physiological processes. An increasing body of evidence indicates the important role of the glutamatergic system in the pathophysiology of depression. Firstly, depressed patients exhibit elevated levels of glutamate both in plasma and the limbic

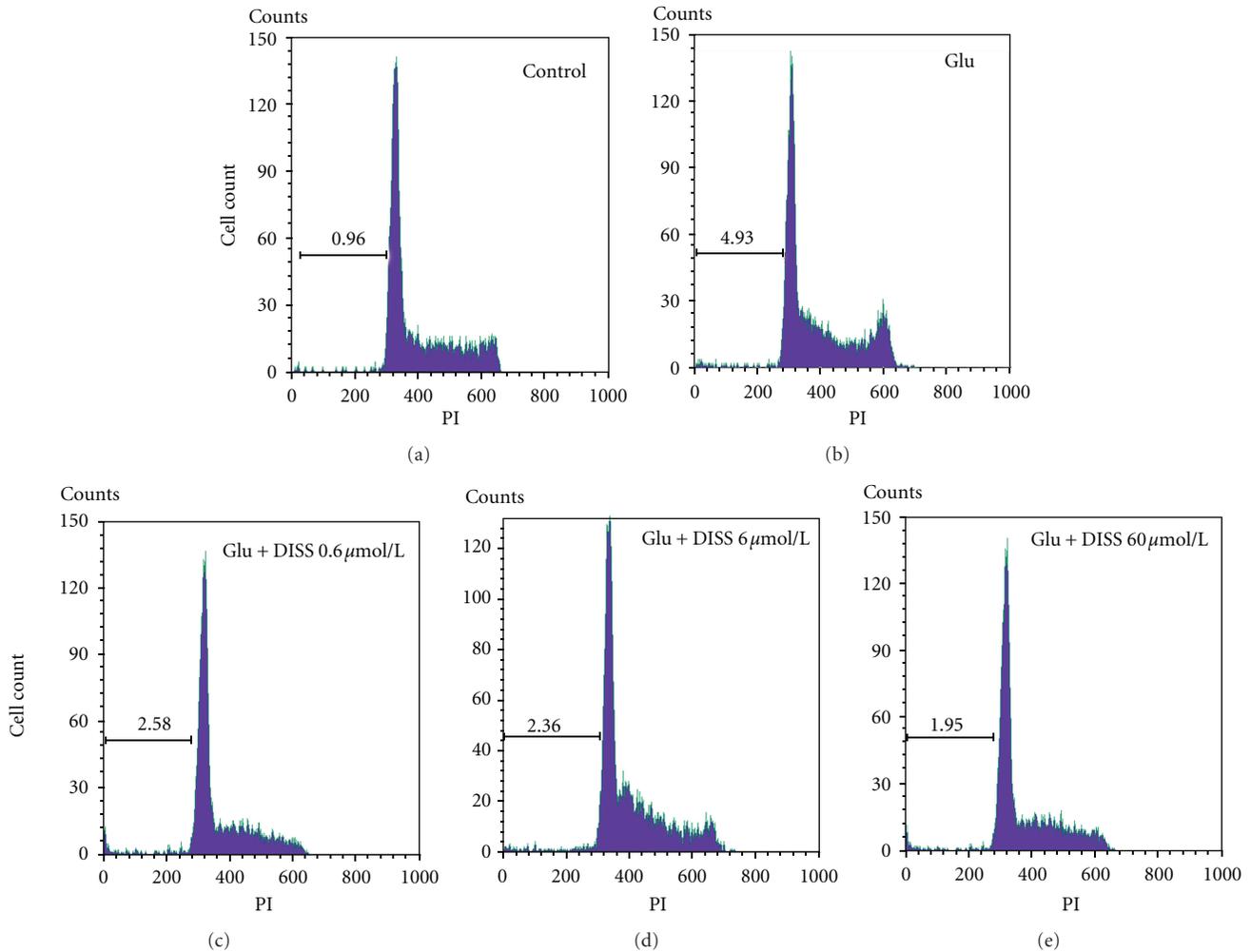


FIGURE 2: Effect of DISS against glutamate- (Glu-) induced neurotoxicity in cultured SH-SY5Y cells by flow cytometric DNA analysis. The sub-G1 peaks were determined by flow cytometry. (a) Control, (b) Glu alone, (c) Glu + 0.6 $\mu\text{mol/L}$ DISS, (d) Glu + 6 $\mu\text{mol/L}$ DISS, (e) Glu + 60 $\mu\text{mol/L}$ M DISS. Bar(|—|) represents a sub-G1 or hypodiploid DNA fraction.

TABLE 1: Effect of DISS on Bax and Bcl-2 expression in SH-SY5Y cells after Glu exposure. β -actin was used as an internal control. Results are from three independent experiments.

	Control	Glu (8 mM)	Glu + DISS (8 mM + 0.6 $\mu\text{mol/L}$)	Glu + DISS (8 mM + 6 $\mu\text{mol/L}$)	Glu + DISS (8 mM + 60 $\mu\text{mol/L}$)
Bax	1 \pm 0.249	2.317 \pm 0.280 ^{##}	2.081 \pm 0.316	1.921 \pm 0.458	1.597 \pm 0.351 ^{**}
Bcl-2	1 \pm 0.109	0.467 \pm 0.025 ^{##}	0.631 \pm 0.018	0.743 \pm 0.028	0.825 \pm 0.016 ^{**}

^{##} $P < 0.01$, compared with normal cultures; ^{*} $P < 0.05$, ^{**} $P < 0.01$ compared with Glu alone group.

brain areas, which are believed to be involved in mood disorders [11]. Additionally, it has been shown that chronic treatment with antidepressants of different mechanisms reduces the glutamate release in rats [12, 13].

DISS is the active oligosaccharide ester component and has been proven to have antidepressant [5, 6], cerebral protective, and cognition-improving effects [14]. In the present study, we demonstrated that cotreatment with DISS protected SH-SY5Y neuronal cells against glutamate insult, with maximal neuroprotection being observed at 60 $\mu\text{mol/L}$.

Similarly, flow cytometry detects apoptotic cells with fragmented nuclei, which are also called sub-G1 cells. DISS treatment significantly reduced the apoptotic cells induced by Glu. We also investigated whether DISS has any effect on the transcriptional level of Bax and Bcl-2 in Glu-treated cells. Proapoptotic gene Bax, antiapoptotic gene Bcl-2 mRNA expression levels were measured by Real-Time PCR. It was reported that Bax and Bcl-2, the two main members of Bcl-2 family, play a key role in the mitochondrial pathway of apoptosis. Bax has been implicated in promoting cell

apoptosis, whereas Bcl-2 is inhibiting apoptosis [15, 16]. Our results indicate that DISS provides neuroprotection partly by the dramatic inhibition of bax overexpression induced by glutamate and increasing antiapoptotic bcl-2 gene expression.

In summary, DISS could provide neuroprotection in glutamate-induced cell injury model. The protective effects of DISS were related to modulating apoptosis-related gene expression, by downregulating the synthesis of proapoptotic Bax and upregulating antiapoptotic bcl-2 expression. Further studies on the neuroprotection effects of DISS in primary neuronal cell injury model, mediated via MAPK-CREB-BDNF/TrkB signaling pathway, are currently under way to evaluate the detail neuroprotective mechanism of therapeutic efforts of DISS.

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