

Original Article

Neurotoxicity of *Veratrum nigrum* L. and the molecular mechanism of veratridine toxicity

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Abstract: *Veratrum nigrum* L. has been used as an herbal preparation in worldwide for treatment of blood-stroke, excessive phlegm, epilepsy, etc. Veratridine is both active and toxic constituents of *Veratrum nigrum* L., which has been reported to affect excitable membranes, peripheral nerves, and skeletal and cardiac muscle. Previous work and literature research are reported that veratridine can act on the central nervous system. Therefore, it is very meaningful to study the neurotoxicity of veratridine. Cell viability, LDH release, ROS generation, MMP expression, Ca²⁺ concentration and apoptosis were measured to evaluate the neurotoxic mechanism of veratridine in SH-SY5Y cells. A positive injury model was established with glutamic acid in SH-SY5Y cells and Western blot was used to correlate toxicity with the MAPK signaling pathway. The results show that veratridine causes, MMP expression changes, Ca²⁺ accumulation in the cytoplasm, increased ROS and LDH releasing and SH-SY5Y cell membrane damage. SH-SY5Y cell viability diminished due to apoptosis. The MAPK signaling pathway is activated by veratridine, and driving forward apoptosis. The study provided evidence of the neurotoxic caused by veratridine and revealed the molecular mechanism behind its neurotoxic effects.

Keywords: *Veratrum nigrum* L., veratridine, SH-SY5Y cells, neurotoxicity, toxicity mechanism, MAPK

Introduction

Traditional Chinese medicines are gaining increasing popularity worldwide for disease treatment. Though the herb *Veratrum nigrum* L. has been demonstrated to have toxic side effects, it has been used as an herbal preparation in China, Japan, Southeast Asia, and by native Americans for the treatment of hemorrhagic stroke, excessive phlegm, epilepsy, etc [1, 2]. *Veratrum* has been documented to be toxic to the digestive tract mucosa, the dorsal nucleus of the vagus nerve, and the central nervous system [3, 4]. Modern phytochemical and pharmacological studies have shown that both the active and toxic constituents are alkaloids, and some studies suggest that it is mutagenic and teratogenic [5, 6]. We previously reported that the LD₅₀ of aqueous extracts of *Veratrum nigrum* L. is 2.566 g/kg after intragastric administration, and that mice die 5-10 min after drug

administration accompanied by convulsions [7, 8].

Veratridine (Ver), which accounts for 33% of the total plant alkaloid content, is considered to be toxic and has been reported to affect excitable membranes, peripheral nerves, and skeletal and cardiac muscle [9-12]. Ver increases intracellular Na⁺, which subsequently increases intracellular Ca²⁺ via Na⁺-Ca²⁺ exchange, Low doses of Ver alkaloids have hypotensive effects, while high doses can be neurotoxic [13, 14]. It has also been reported that Ver can act on the central nervous system and modify opioid and muscarinic binding sites in brain slices. The brain can be excited after these inhibitory effects, leading to disturbances of consciousness such as convulsions, drowsiness, and coma [15, 16]. The human SH-SY5Y neuroblastoma cell line has similarities to nerve cell morphology, physiology, and biochemical function,

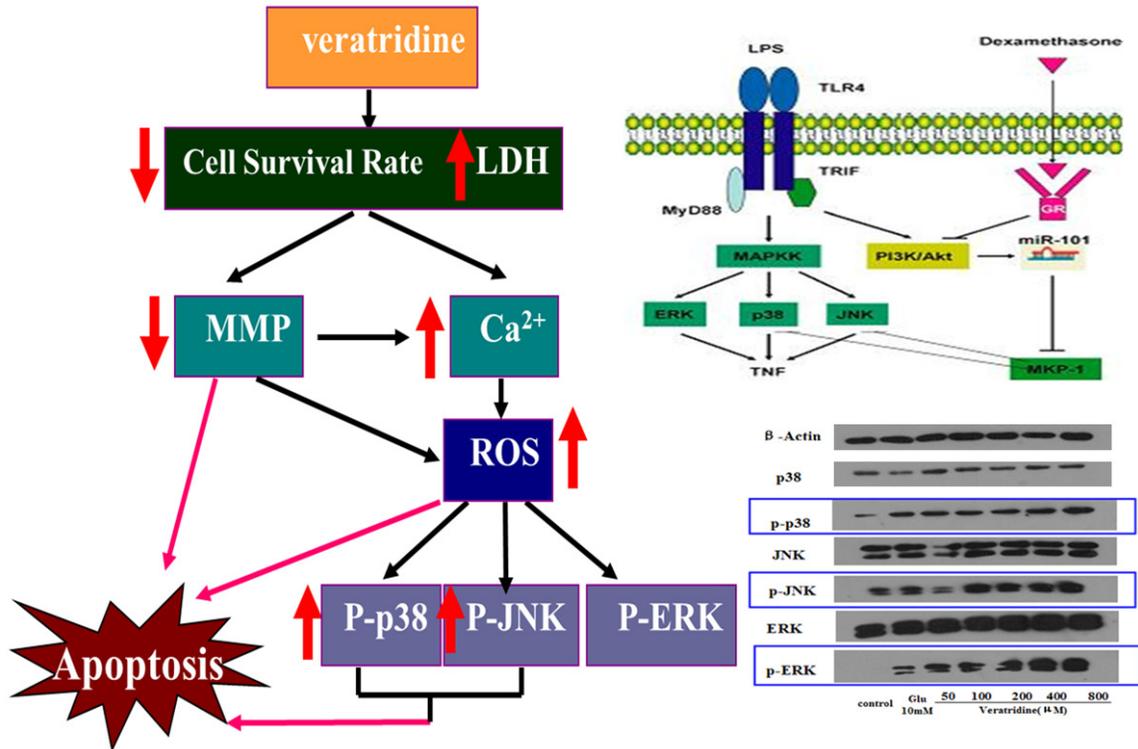


Figure 1. Graphical abstract, The inner structure of the paper.

and is used to model neurodegenerative diseases [17]. We documented differentially expressed genes in SH-SY5Y cells treated with Ver using a cDNA microarray. These differentially expressed genes are involved with metabolism, mitochondrial function, the cell cycle, cell differentiation, cell signaling, and apoptosis, and their disturbance results in subacute or chronic injury to the nervous system [18]. Therefore, it is very meaningful to study the neurotoxicity of Ver.

Apoptosis is involved in acute neurotoxicity and chronic neurodegenerative disorders, so drugs that block this type of cell death are of interest. Apoptosis is initiated by Ver-mediated oxidative stress, which damages the membrane and causes lactate dehydrogenase (LDH) release and decreased mitochondrial membrane potential (MMP). Mitochondrial dysfunction is a significant source of reactive oxygen species (ROS) [19, 20].

Mitogen-activated protein kinase (MAPK) signaling pathways are composed of Ser/Thr protein kinases that activate as a cascade. MAPKs respond to diverse cellular and extracellular

stimuli such as growth factors, cytokines, extracellular mitogens, and stress [21, 22]. MAPKs are involved in cell differentiation, proliferation, and programmed cell death. Four pathways of MAPK signaling transduction have been identified, ERK1/2, JNK, P38, and ERK5, among which p38/JNK/MAPK signaling transduction is important in cell differentiation [23-25]. At present, the activation of ERK signaling is reported to promote cell proliferation, while JNK signaling is tied to neurodegenerative or neurological diseases [26, 27]. Both p38 and JNK signaling pathways are associated with apoptosis. Thus, we assessed mitochondrial dysfunction-induced neurotoxicity in SH-SY5Y cells and observed the mechanisms underlying neurotoxicity after treatment with the toxin Ver. Abstract mining the inner structure of the graph, as shown in **Figure 1**.

Materials & methods

Reagents

Veratridine (Ver) and Glutamic acid (Glu) (purity > 99%) was obtained from the National Institute for the control of Pharmaceutical and Biological

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Product (Beijing, China). RPMI-1640 medium and fetal bovine serum were purchased from Gibco (Carlsbad, CA). Dichlorofluorescein diacetate (DCFH-DA), lactate dehydrogenase (LDH) and a Cell Counting Kit-8 (CCK-8) were purchased from Sigma (St. Louis, MO). An annexinV-fluorescein isothiocyanate (FITC)-propidium iodide (PI) double-stained apoptosis detection kit was obtained from Biosea biotechnology (Beijing, China). Fluo-3-acetoxymethyl ester (Fluo-3-AM) and Hoechst 3325 were purchased from Invitrogen Biotechnology (Carlsbad, CA). The ROS fluorescent probe dihydroergotamine (DHE) was purchased from Vigorous Biotechnology Beijing (Beijing, China). Polyvinylidene difluoride membrane (PVDF) and an ECL kit were purchased from Amersham (Arlington Heights, IL). Primary antibodies to β -actin (1:500), ERK1/2 (1:500), p-ERK1/2 (1:500), c-Jun NH2-terminal kinase (JNK) (1:500), p-JNK (1:200), P38 (p38 MAPK) (1:400), p-P38 (1:200), and secondary antibody horseradish peroxidase (HRP) conjugated immunoglobulin G (IgG) (1:2,000) were purchased from Santa Cruz biotechnology (Santa Cruz, CA). Other analytically pure reagents were from Promega.

Cell culture and treatment

SH-SY5Y human neuroblastoma cells were grown in RPMI-1640 supplemented with 15% fetal calf serum. Cells were maintained at 37°C in an incubator with a saturated humid atmosphere containing 95% air and 5% CO₂. Cells were passaged once every three days. All experiments were conducted on cells between passages 10-20 after cells were purchased from Peking Union Medical College Cell Bank (Beijing, China).

Cell viability analysis

Cytotoxicity was assayed in SH-SY5Y cells plated in 96-well plates (1.5×10⁵ cells/ml) overnight and replaced with serum-free medium supplemented with Glu for 16 h or Ver for 48 h. 10 μ L of CCK-8 was added to each well and incubated for 1-4 h. Absorbance was read at 450 nm with a Beckman Victor X Microplate Reader (Beckman, Foster City, CA). Reductions in optical density (OD) due to drug treatment were used to assess cell viability and normalized to controls incubated in medium (100% viable).

LDH release

LDH release was measured using a commercial kit. SH-SY5Y cells were plated at 1.5×10⁵ cells/ml in 96-well plates, with indicated concentrations of Glu for 16 h and Ver for 48 h. We ran the reaction in the dark for 10 min prior to measurement, and the absorbance was read at 450 nm using a microplate reader. Results are expressed as percent relative to controls.

MMP measurement

MMP was measured using flow cytometry and the mitochondrial-specific cationic dye JC-1. SH-SY5Y cell (1.5×10⁵ cells/ml) suspensions were plated in 12-well plates with Glu for 16 h or Ver for 48 h. Cells were harvested, washed twice, and incubated with 0.5 mL JC-1 (25 μ M) for 20 min at 37°C. MMP was assayed and green (JC-1 monomer) and red (JC-1 aggregate) fluorescence were monitored at an emission wavelength of 525 nm and 595 nm. Changes in ratios between measurements indicated MMP changes.

Intracellular ROS and Ca²⁺ measurements

SH-SY5Y cell (1.5×10⁵ cells/ml) suspensions were plated in 12-well plates with the indicated concentrations of Glu for 16 h and Ver for 48 h. Intracellular ROS and cytosolic Ca²⁺ were measured using the fluorescent probe DCFH-DA and Fluo-3-AM, respectively, and a fluorescent-activated cell sorter. DCFH-DA is converted to a fluorescent compound in the presence of ROS. Fluo-3/AM was added to treated cells to measure Ca²⁺. After treatment with the indicated drugs, cells were incubated with DCFH-DA (10 μ M) for 20 min at 37°C in the dark. (ROS assay) and Fluo-3/AM (5 μ mol/L) for 30 min at 37°C (Ca²⁺ assay), and cells were harvested and suspended in 500 μ L HBSS. Intracellular ROS and Ca²⁺ were measured using a flow cytometer (excitation wavelength 488 nm; emission wavelength 535 nm).

Cell apoptosis

Hoechst 33342 was used to assess apoptosis in SH-SY5Y cell (1.5×10⁵ cells/ml). Suspensions were plated in 6-well plates with Glu for 16 h or Ver for 48 h. Then, Hoechst 33342 (5 μ g/mL) was added for 10 min at 37°C. Apoptosis was measured under a fluorescent microscope at

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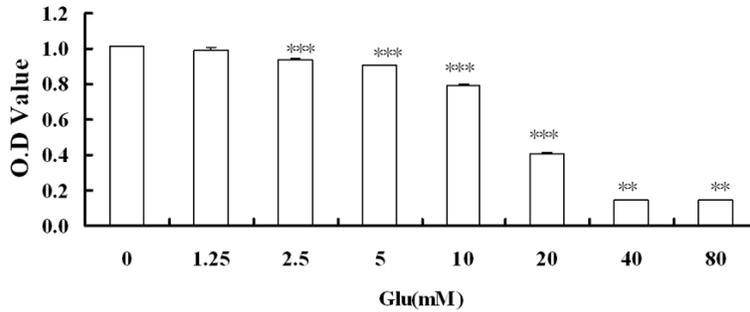


Figure 2. Cytotoxicity of Glu to SH-SY5Y cells. ** $p < 0.01$, *** $p < 0.001$ vs. control group. (1): Control; (2): Glu 1.25 mM; (3): Glu 2.5 mM; (4): Glu 5 mM; (5): Glu 10 mM; (6): Glu 20 mM; (7): Glu 40 mM; (8): Glu 80 mM.

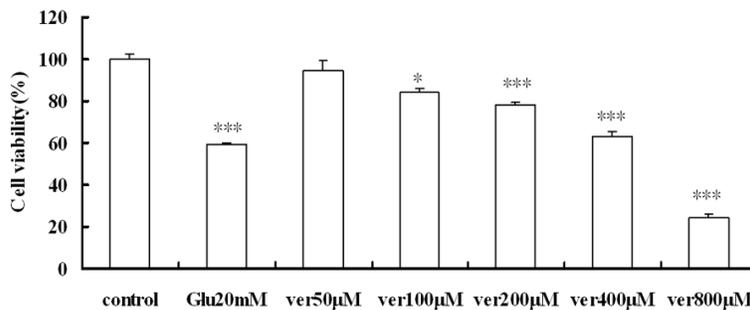


Figure 3. Cytotoxicity of Ver to SH-SY5Y cells. ** $p < 0.01$, *** $p < 0.001$ vs. control group. (1): Control; (2): Glu 20 mM; (3): Ver 50 μ M; (4): Ver 100 μ M; (5): Ver 200 μ M; (6): Ver 400 μ M; (7): Ver 800 μ M.

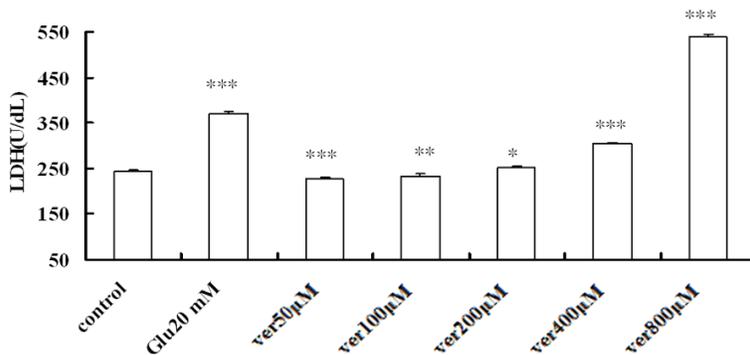


Figure 4. Ver increases LDH release in SH-SY5Y cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group. (1): Control; (2): Glu 20 mM; (3): Ver 50 μ M; (4): Ver 100 μ M; (5): Ver 200 μ M; (6): Ver 400 μ M; (7): Ver 800 μ M.

340 nm (CKX41, Olympus, Tokyo, Japan). Apoptosis was also measured using AnnexinV-FITC and PI. SH-SY5Y cells were plated (1.5×10^5 cells/ml) in 12-well plates with indicated concentrations of Glu for 16 h or Ver for 48 h. Cells were harvested and washed twice in ice-cold PBS and suspended in 200 μ L ice-cold binding buffer. Then, 10 μ L horseradish peroxidase FITC-labeled AnnexinV and 5 μ L PI were added. The cell suspension was gently mixed and incu-

bated in the dark for 15 min at room temperature. Apoptosis was measured using flow cytometry (488 nm excitation). Fluorescent intensity was measured (530 nm emission). AnnexinV+/PI- was used to document early apoptosis and AnnexinV+/PI+ confirmed late apoptotic stages or necrotic cells.

Western blot

Proteins from SH-SY5Y cells were isolated and measured, and densitometric analysis was performed with Gel-Pro Analyzer Software version 3.0. Cells were washed twice in PBS and suspended in lysis buffer for 30 min at 4°C. Supernatants were collected after centrifugation at $15,000 \times g$ for 20 min at 4°C. Lysates (20 μ g) were resolved with 10% SDS-PAGE, and fractionated proteins were electrophoretically transferred to an immobilon PVDF membrane and probed with primary antibodies and secondary antibody of horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) (1:2,000). Blots were developed with ECL and reprobated with anti-actin antibody as a protein loading control.

Statistical analysis

All experiments were repeated three times and data are expressed as means \pm SD. Statistical analyses were performed using one way ANOVA. Statistical significance was tested by SPSS software and p -values < 0.05 were considered statistically significant.

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Results

SH-SY5Y cells were less viable after exposure to Ver (Figures 2 and 3). Glu was used as a damage model drug for cell injury in a cell dam-

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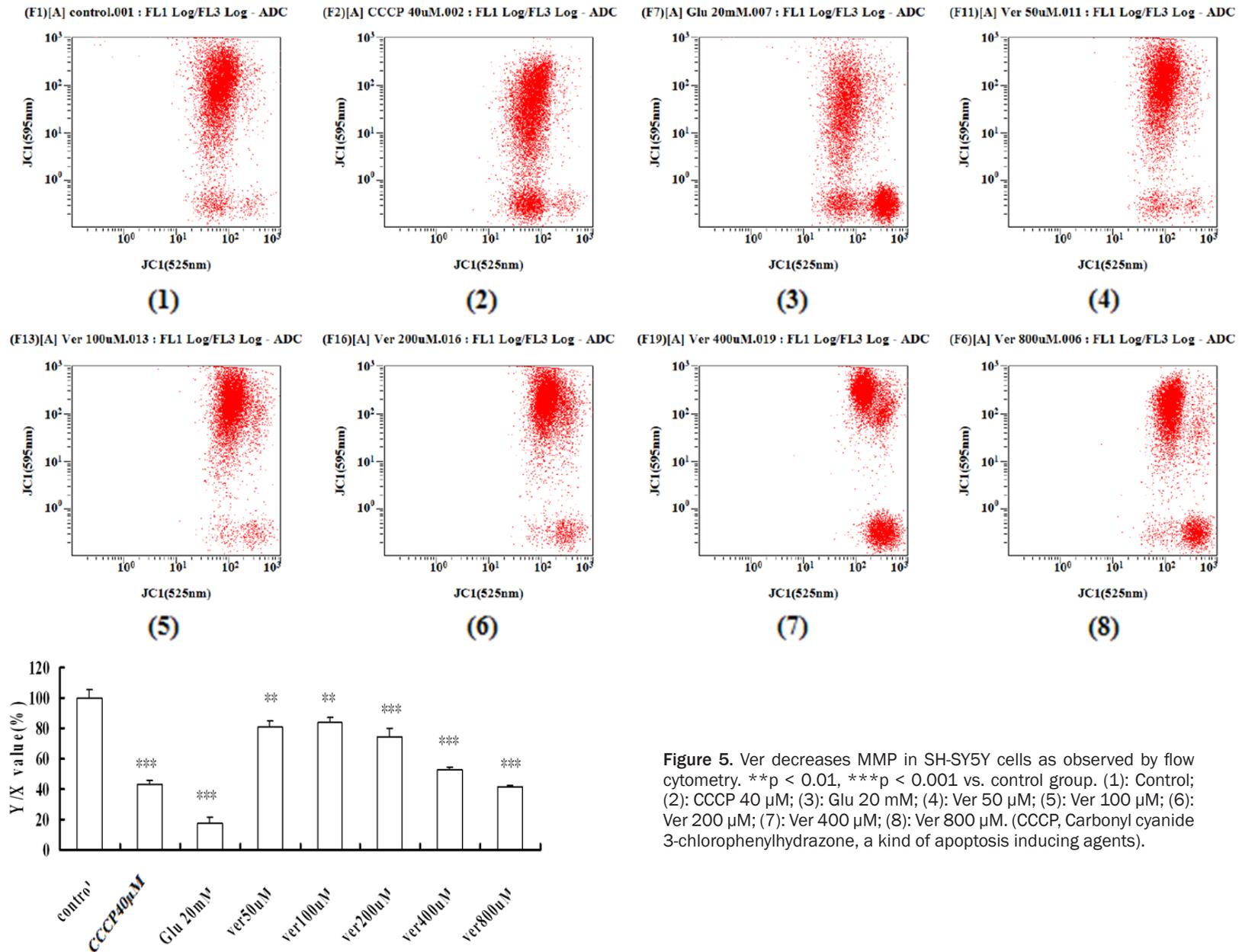


Figure 5. Ver decreases MMP in SH-SY5Y cells as observed by flow cytometry. **p < 0.01, ***p < 0.001 vs. control group. (1): Control; (2): CCCP 40 μM; (3): Glu 20 mM; (4): Ver 50 μM; (5): Ver 100 μM; (6): Ver 200 μM; (7): Ver 400 μM; (8): Ver 800 μM. (CCCP, Carbonyl cyanide 3-chlorophenylhydrazone, a kind of apoptosis inducing agents).

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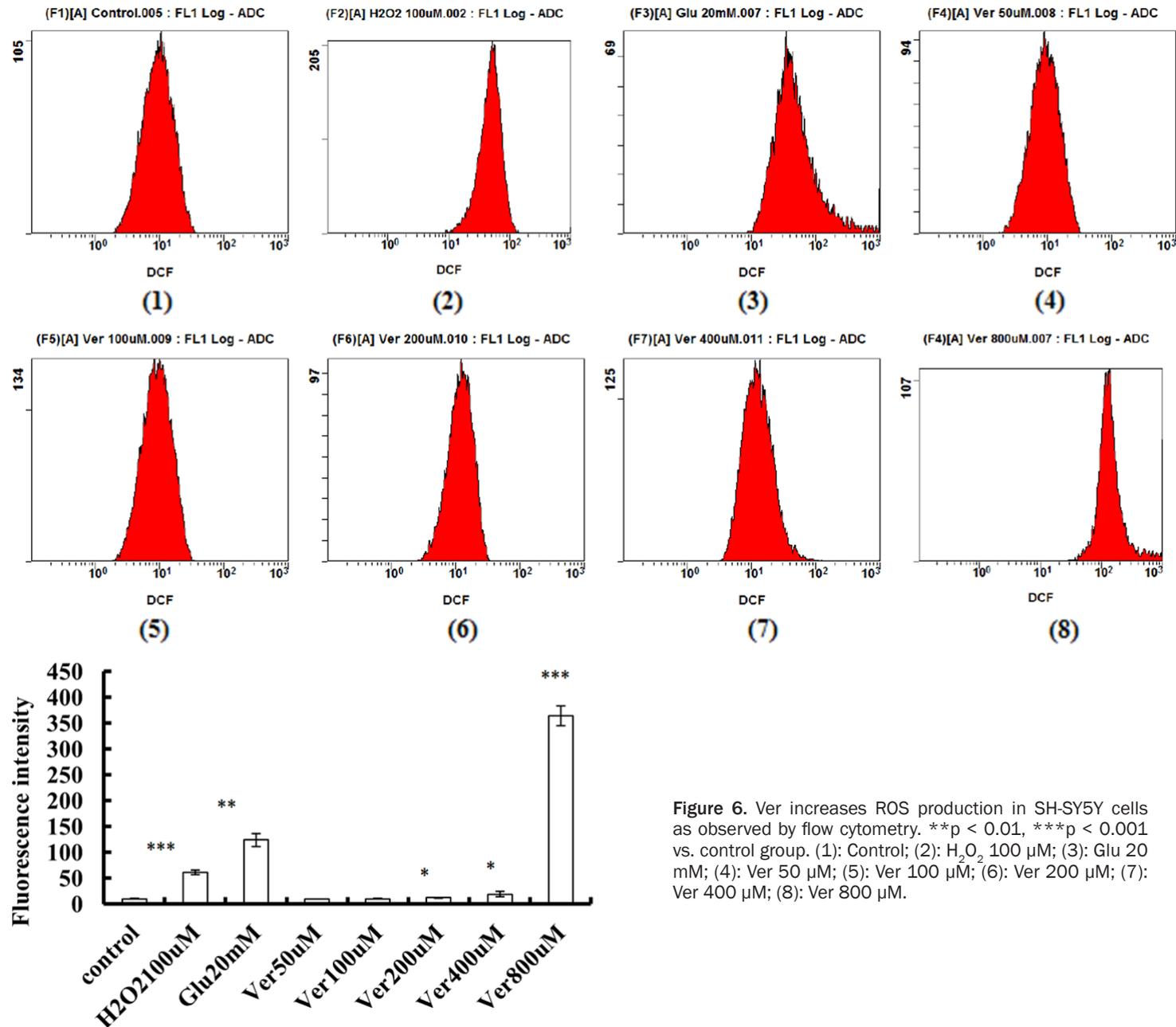


Figure 6. Ver increases ROS production in SH-SY5Y cells as observed by flow cytometry. **p < 0.01, ***p < 0.001 vs. control group. (1): Control; (2): H₂O₂ 100 μM; (3): Glu 20 mM; (4): Ver 50 μM; (5): Ver 100 μM; (6): Ver 200 μM; (7): Ver 400 μM; (8): Ver 800 μM.

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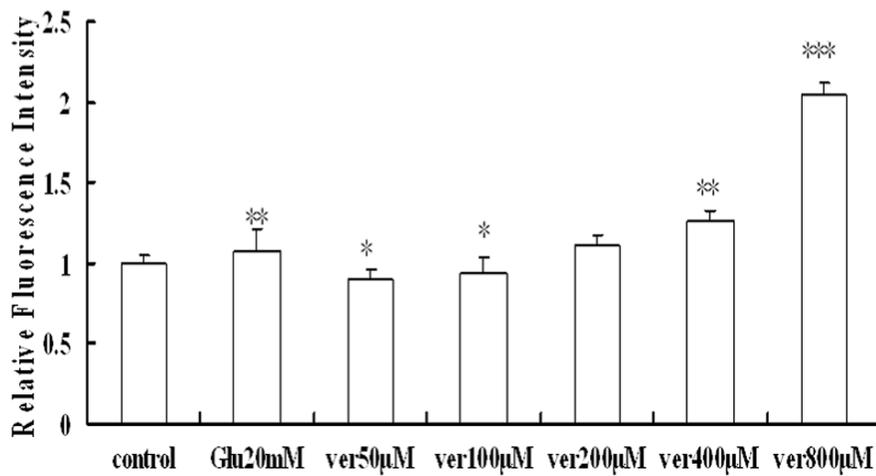
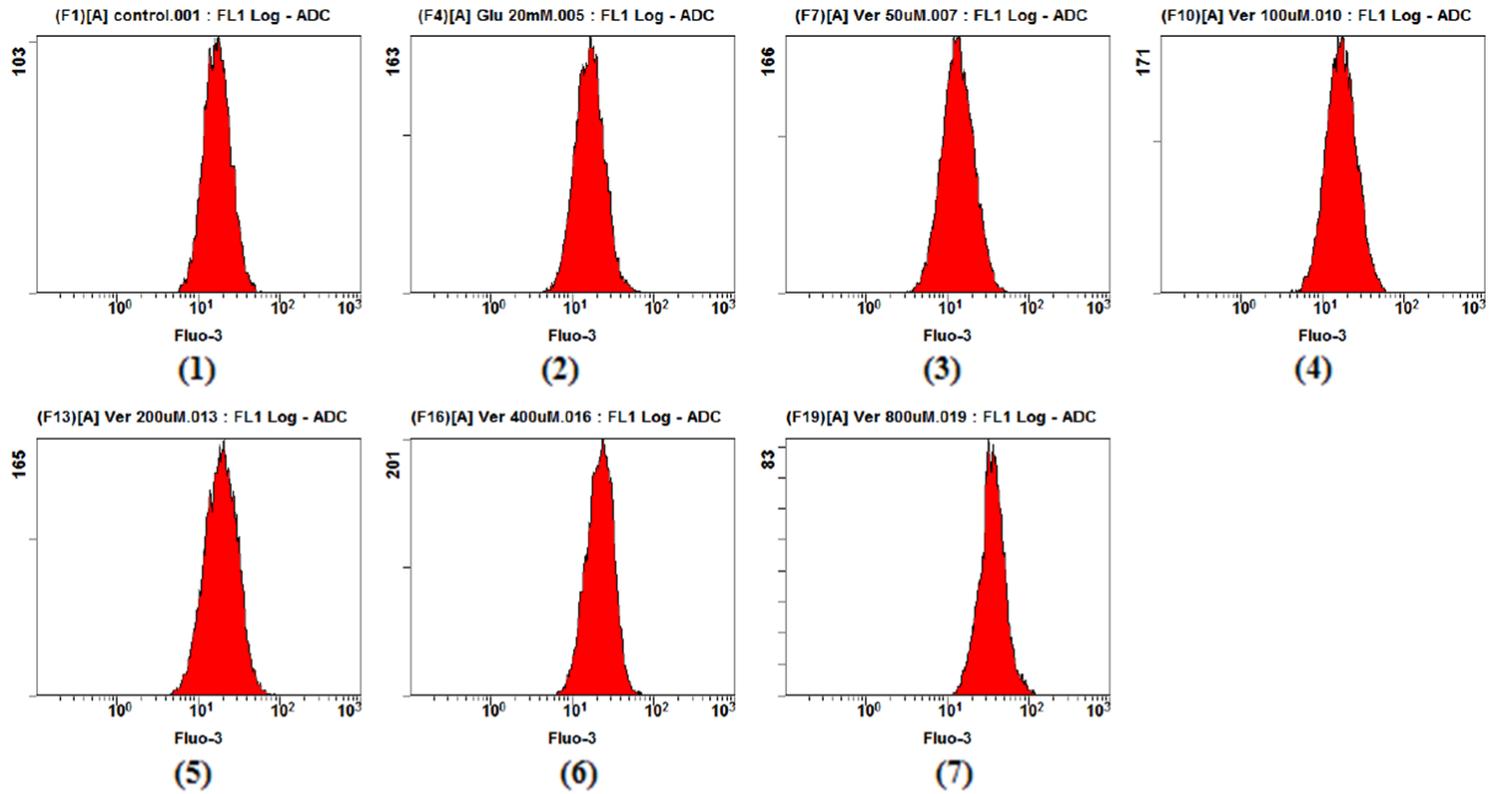


Figure 7. Ver increases Ca²⁺ accumulation in SH-SY5Y cells as observed by flow cytometry, **p < 0.01, ***p < 0.001 vs. control group. (1): Control; (2): Glu 20 mM; (3): Ver 50 μM; (4): Ver 100 μM; (5): Ver 200 μM; (6): Ver 400 μM; (7): Ver 800 μM.

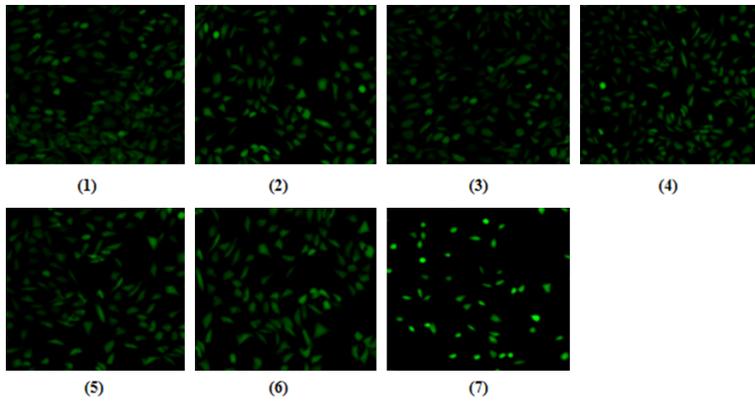


Figure 8. Ver induces apoptosis as observed by fluorescence microscopy 100×. (1): Control; (2): Glu 20 mM; (3): Ver 50 μM; (4): Ver 100 μM; (5): Ver 200 μM; (6): Ver 400 μM; (7): Ver 800 μM.

age model (**Figure 2**), Glu decreased cell survival in a dose-dependent manner; cell survival rapidly decreased at 20 mM and reached a maximum at 40 mM. Thus, a positive damage model of SH-SY5Y cells was established using Glu 20 mM for 16 h. **Figure 3** showed that Ver stimulation decreased cell viability in a dose-dependent manner across a range of 50-800 μM. The IC_{50} value for Ver was calculated to be 439 μM. LDH release due to cell damage was measured (**Figure 4**), and Ver increased LDH release in dose-dependent manner. The MMP reflects mitochondrial function, and apoptosis is triggered when MMP decreases sufficiently. JC-1 is a fluorescent probe used to measure MMP and we noted that at high MMP, JC-1 accumulated in the mitochondrial matrix and formed polymers that fluoresced red. When MMP was low, JC-1 monomers fluoresced green. Changes in red-to-green fluorescence were used to represent MMP. **Figure 5** showed that SH-SY5Y cells treated with Ver for 48 h had reduced MMP.

Ver-Induced intracellular ROS production and Ca²⁺ measurement in SH-SY5Y cells

ROS was measured with DCFH-DA, and flow cytometry was used to assess intracellular fluorescent intensity. **Figure 6** showed that with Ver exposure at 50-400 μM, ROS did not increase significantly, but at 800 μM, ROS production increased significantly. Mitochondria help regulate Ca²⁺ homeostasis. **Figure 7** showed that with increasing Ver, the Ca²⁺ mitochondrial function was altered; which in comply with significantly decreased MMP resulting in mitoch-

ondrial Ca²⁺ overload and the induction of ROS. High ROS causes feedback regulation of Ca²⁺ release from the intracellular calcium pool causing intracellular Ca²⁺ accumulation.

Ver induced apoptosis in SH-SY5Y cells

Hoechst 33342 staining was used to distinguish the manner of cell death and as shown in **Figure 8**, cell volume decreased, cell connections were reduced, and cytoplasmic density increased. Flow cytometric

data indicated that (**Figure 9**) apoptosis increased after treatment with 100-800 μM Ver for 48 h.

Western blot

MAPK pathways were activated by Ver and linked to apoptosis. We measured ERK, c-Jun, JNK, and p38 activity in SH-SY5Y cells. Blots in **Figure 10** showed that p-P38, p-JNK, and p-ERK protein increased after treatment with Ver, indicating pathway activity. Activation of p-P38, p-JNK, and p-ERK signaling pathways can aggravate nerve injury in a dose-dependent manner. p-ERK did not change significantly, indicating that Ver partly increased activation of ERK. Co-incubation with Ver and p38 inhibitor SB203580 or a JNK inhibitor SP600125 decreased the phosphorylation levels of p38 and JNK (**Figure 11**). Pretreatment of cells with the p38 inhibitor SB203580 or the JNK inhibitor SP600125 significantly improved SH-SY5Y cell viability, suggesting that inhibiting these signaling pathways reduced apoptosis.

Discussion

Alkaloids in *V. nigrum L.* are toxic, as is the herbal product derived from its roots and rhizomes. Previous work by our laboratory found that the LD_{50} of aqueous extracts of *Veratrum nigrum L.* was 2.566 g/kg after intragastric administration, and mice died 5-10 min after drug administration. Toxicity manifested predominantly as trembling, convulsions, and spasms, but the major organs showed no obvious

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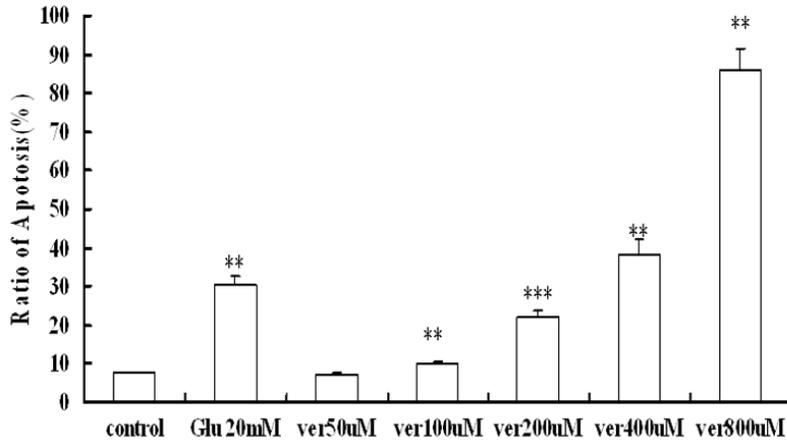
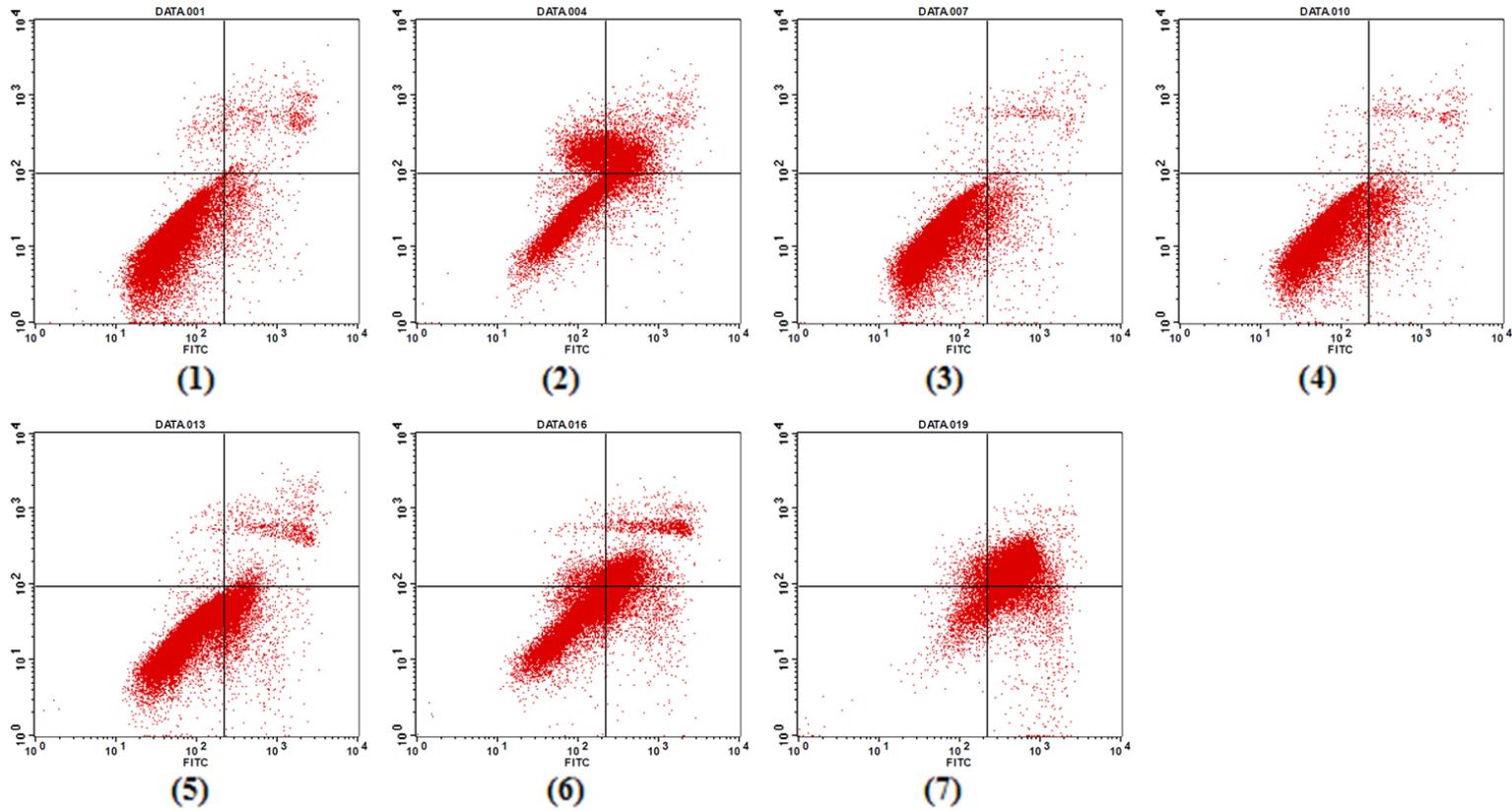


Figure 9. Ver affects SH-SY5Y cell viability as observed by flow cytometry. **p < 0.01, ***p < 0.001 vs. control group. (1): Control; (2): Glu 20 mM; (3): Ver 50 μ M; (4): Ver 100 μ M; (5): Ver 200 μ M; (6): Ver 400 μ M; (7): Ver 800 μ M.

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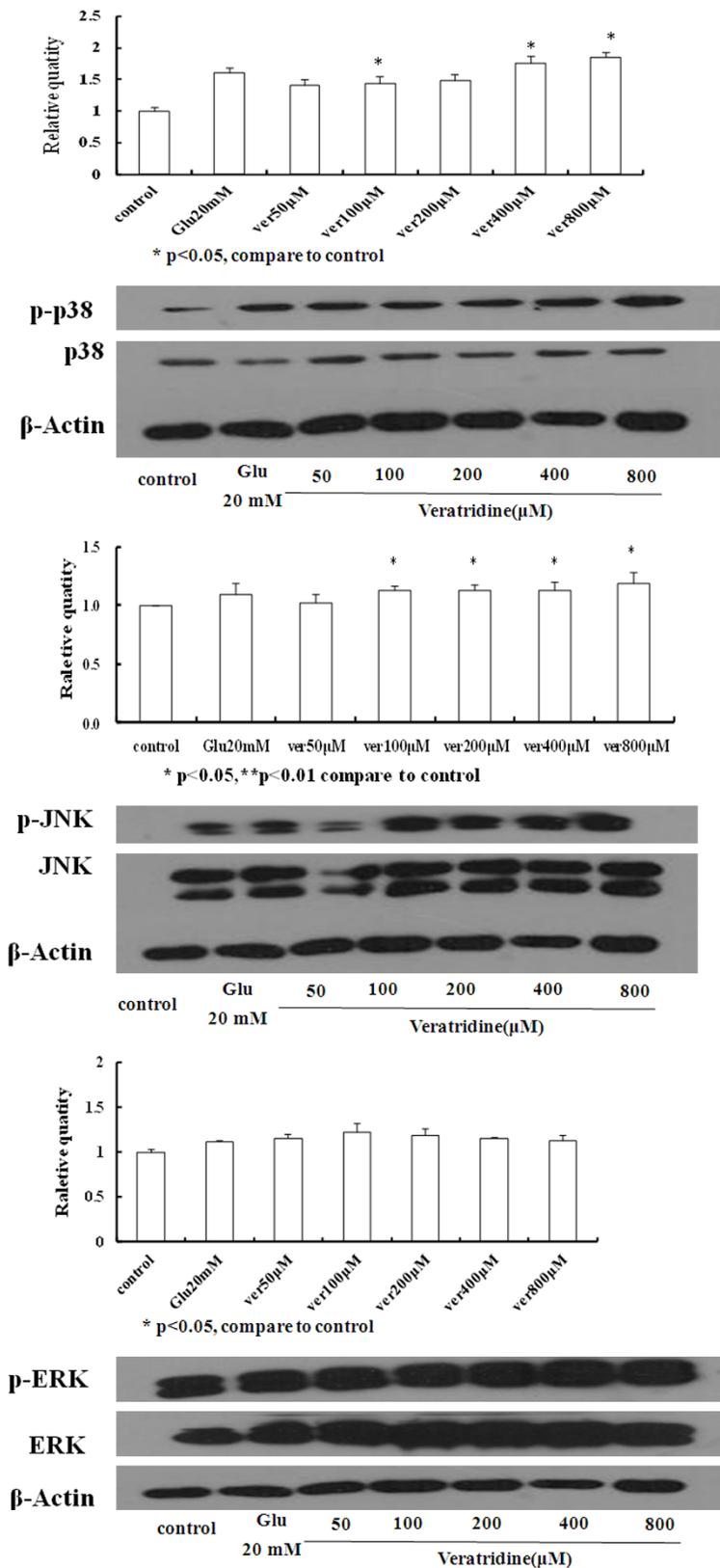


Figure 10. Ver increases the expression of p38, JNK, and ERK kinases related to MAPK signal transduction in SH-SY5Y cells.

lesions at necropsy by the naked eye. Based on that study and the guidelines for Chinese natural medicine acute toxicity testing, we speculate that the toxicity was caused predominantly by suppression of the central nervous system. *Veratrum nigrum* L. water extracts exert toxic effects on the digestive tract mucosa, nucleus nervi vagi, and central nervous system [28]. Pharmacological studies of veratrum alkaloids have been performed previously and found that these alkaloids stimulate the central nervous system and inhibit brain function. This results in spasms, convulsions, coma, drowsiness, and other neurological symptoms in animals, which is consistent with the symptoms found in mice in previous studies [29, 30].

Ver is the toxic component of *Veratrum nigrum* L. and has documented toxicity to the CNS and to muscles as well as having mutagenic potential. We previously investigated the differentially expressed genes in SHSY5Y cells treated with Ver using cDNA microarrays. Cells were treated with Ver for 8 h. The differentially expressed genes were involved in metabolism, mitochondrial function, the cell cycle, cell differentiation, cell signaling, and apoptosis

Neurotoxicity is mediated by apoptosis during chronic neurodegenerative diseases, and oxidative stress contributes to this apoptosis. Thus, we measured mitochondrial dysfunction-induced neurotoxicity and elucidated the mechanism of action of mitochondrial damage by Ver in SH-SY5Y cells. Data revealed that Ver treat-

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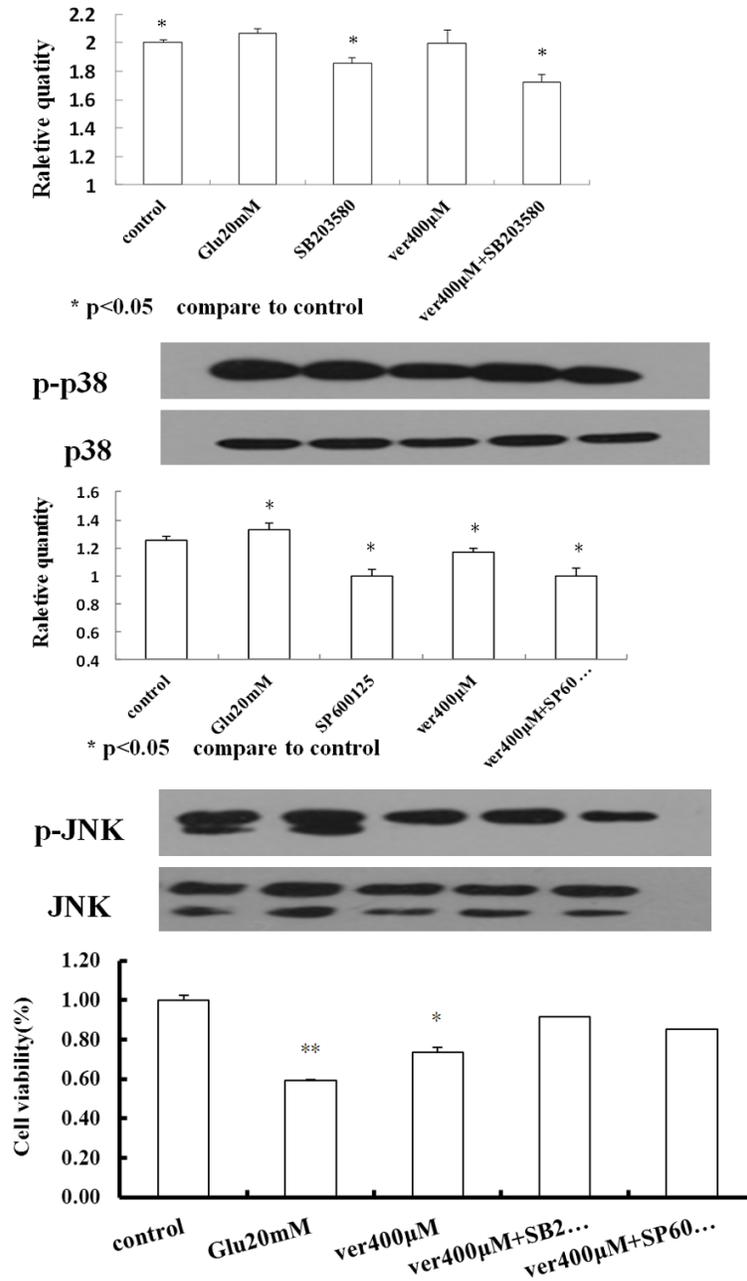


Figure 11. Pretreatment of cells with SB203580 or SP600125 significantly improves SH-SY5Y cell viability, and co-incubation with Ver decreases the phosphorylation levels of p38 and JNK in SH-SY5Y cells.

ment altered the expression of MMP, caused Ca^{2+} concentration overload, increased reactive oxygen species production, enhanced LDH release, damaged the cell membrane, reduced SHSY5Y cell viability, and induced apoptosis. Under oxidizing conditions, the MAPK signaling pathway is activated, which increases apoptosis. We presently found that MAPK signal transduction is associated with

Ver neurotoxicity in SH-SY5Y cells, as it was activated and the expressions of p-p38 and p-JNK kinases were increased. SHSY5Y cells pretreated with the JNK inhibitor SP600125 or the p38 inhibitor SB203580 were more viable, suggesting that these inhibitors partly prevented apoptosis [31-33]. The study illustrates the neurotoxicity of *Veratrum nigrum L.* and reveals the molecular mechanism behind the neurotoxic effects of *Veratrum nigrum L.*

In conclusion, the present study provides additional evidence that the active ingredient of *Veratrum nigrum L.*, veratridine, is neurotoxic. We also revealed the molecular mechanism behind its neurotoxic effects, which consists of alterations in mitochondrial membrane potential leading to the release of ROS, Ca^{2+} overload, and eventual cell death. This study provides an experimental basis for the clinical use of *Veratrum nigrum L.* and poisoning treatment of *Veratrum nigrum L.*

Acknowledgements

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Disclosure of conflict of interest

None.

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