Protective effects of *Dendrobium nobile* Lindl. alkaloids on amyloid beta (25–35)-induced neuronal injury

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**Abstract**

*Dendrobium nobile* Lindl. alkaloids (DNLA), the active ingredients of a traditional Chinese medicine *Dendrobium*, have been shown to have anti-oxidative effects, anti-inflammatory action, and protective effect on neurons against oxygen-glucose deprivation. However, it is not clear whether DNLA reduces amyloid-beta (Aβ)-induced neuronal injury. In this study, cortical neurons were treated with DNLA at different concentrations (0.025, 0.25, and 2.5 mg/L) for 24 hours, followed by administration of Aβ<sub>25–35</sub> (10 μM). Aβ<sub>25–35</sub> treatments increased cell injury as determined by the leakage of lactate dehydrogenase, which was accompanied by chromatin condensation and mitochondrial tumefaction. The damage caused by Aβ<sub>25–35</sub> on these cellular properties was markedly attenuated when cells were pretreated with DNLA. Treatment with Aβ<sub>25–35</sub> down-regulated the expressions of postsynaptic density-95 mRNA and decreased the protein expression of synaptophysin and postsynaptic density-95, all changes were significantly reduced by pretreatment of cells with DNLA. These findings suggest that DNLA reduces the cytotoxicity induced by Aβ<sub>25–35</sub> in rat primary cultured neurons. The protective mechanism that DNLA confers on the synaptic integrity of cultured neurons might be mediated, at least in part, through the upregulation of neurogenesis related proteins synaptophysin and postsynaptic density-95.

**Key Words:** nerve regeneration; *Dendrobium nobile* Lindl. alkaloids; amyloid beta; neurons; synapse; synaptophysin; postsynaptic density-95; cognitive impairment; neuroprotection; neural regeneration

**Introduction**

Alzheimer’s disease (AD) is the most prevalent mental failure in the elderly. The main clinical manifestation of AD includes a widespread functional disturbance of the central nervous system, characterized by progressive memory impairment, cognitive disorder and altered behavior (Schoemaker et al., 2014). The predominant neuropathological features of AD are senile plaques composed of an aberrant accumulation of amyloid beta (Aβ), neurofibrillary tangles and selective degeneration of neurons and their synapses, generally found in the association cortex and temporal lobe (Zhao et al., 2010; Takeda et al., 2014). It is believed that synaptic injury occurs at a stage prior to the neuronal injury in AD brain (Götz and Ittner, 2008). In addition, the amount of
synaptic loss and synaptic dysfunction is strongly associated with the degree of cognitive dysfunction (Arendt, 2009).

This suggests that reducing synaptic injury would be an important target for early therapy of AD. Maintaining the morphology and function of synapses depends on the integrity of presynaptic and postsynaptic structures (Luo, 2010). Previous studies have demonstrated that an AD brain contains not only a decrease in density and morphological change of dendritic spines, but also a lower expression of synaptophysin (SYP), postsynaptic density (PSD) and other synapse-associated proteins (Jin and Garner, 2008). Improving the expression of these synapse-associated proteins may preserve the synaptic connections, potentially relieving memory and cognitive impairment. Recently, many investigators have suggested that the overexpression of Aβ, the initiator of a cascade reaction of AD, can induce AD-related synaptic dysfunction at an early stage of the disease. This disturbs neurogenesis, and eventually leads to cognitive problems (Senthilkumar et al., 2013; Welzel et al., 2014).

Dendrobium nobile Lindl. alkaloids (DNLA) are alkaloids from a traditional Chinese herbal medicine Dendrobium, which is used to treat cataracts and throat inflammation (Zhang et al., 2003). The major active pharmacological ingredients have been reported to be alkaloids, stilbenoids, glycosides and polysaccharides (Pan et al., 2013). Dendrobium extracts possess a broad spectrum of pharmacological activities, including anti-aging, anti-free radical injury, anti-inflammatory and immunomodulatory effects, and anti-tumor function (Wei, 2005). Our previous research had demonstrated that DNLA has neuroprotective effects. DNLA can inhibit lipopolysaccharide-induced astrocyte activation and reduce the production of pro-inflammatory factors such as interleukin-6 and tumor necrosis factor-α (Zhang et al., 2011). In addition, DNLA can significantly protect against oxygen-glucose deprivation/reperfusion (OGD/RP) induced neuronal damage in rat primary neuron cultures (Wang et al., 2010). In this study, we used Aβ1-42 to disrupt synaptic function, mimicking the early stages of AD, to further investigate the neuroprotective function of DNLA on a model of Aβ-induced neurotoxicity in rat primary neuron cultures. We measured the expression of SYP and postsynaptic density-95 (PSD-95), which may be involved in the mechanism of DNLA-elicited neuroprotective function.

Materials and Methods

Animals
One-day-old Sprague-Dawley rats (specific-pathogen-free II, 30 females and 10 males, aged 6–7 weeks, weighing 180–200 g) were purchased from the Animal Center of the Third Military Medical University of China (certificate No. SCXK (Jun) 2007-0005). The rats were maintained in an air-conditioned animal facility at 23 ± 1°C and a 12-hour light/dark cycle. Rats were given free access to water and food. The experiment was carried out in strict accordance with the State Committee of Science and Technology of the People’s Republic of China Order on November 14, 1988 (revised 2011), and the study protocol followed the Regulations of the 27th August 2007 approved by the Animal Experimental Ethics Committee of the Zunyi Medical University of China.

Cell culture and identification
Primary neuronal cultures were prepared from the cortex of 1-day-old Sprague-Dawley rat pups by enzymatic digestion (Li et al., 2013). Cells dissociated from the cerebral cortices were collected in Dulbecco’s modified eagle medium/nutrient mixture F12 (DMEM/F12; Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO, USA), 10% horse serum (Gibco BRL), and 100 U/mL penicillin/streptomycin. The cells were plated at a density of 1 × 10^4/mL, seeded in 24- and 6-well culture plates pre-coated with poly-D-lysine (Sigma Chemical Co.). Cultures were maintained at 37°C in a humidified incubator of 5% CO₂ and 95% air. After seeding for 24 hours, cultures were replaced with maintenance medium containing Neurabasal-A (Gibco BRL) supplemented with 2% B27 (Gibco BRL). The cortical neurons were immunostained with an antibody against neuron-specific enolase (NSE) (Wuhan Boster Biological Technology, Wuhan, China) and verified by morphologic examination.

Drug intervention
The 7-day-old neuronal cell cultures were used for drug treatments. Cells were homogeneously distributed between each treatment group. Five groups were set up for cell culture experiments, which were: I Control group (untreated cells: no Aβ), II Aβ treated group (10 μM Aβ25-35 Sigma Chemical Co.) for 24 hours, DNLA pretreated groups (treatment with DNLA at concentrations of 0.025, 0.25, or 2.5 mg/L, III – V respectively, for 24 hours, followed by 10 μM Aβ25-35 induction for 24 hours). DNLA (purity ≥ 86.3%) was provided by the pharmacology laboratory of Zunyi Medical College (Guizhou, China).

Morphological examination
At 24 hours after the administration of Aβ25-35 morphological changes of the cell growth and synaptic density of each group were examined under a light microscopy (Leica Microsystems Ltd., Wetzlar, Germany). The ultrastructure of neurons was examined using a transmission electron microscope (Hitachi, Tokyo, Japan).

Leakage of lactate dehydrogenase (LDH)
At 24 hours after the administration of Aβ25-35, LDH release into the culture medium was detected by biochemically measuring the activity of LDH with a microplate photometer (Thermo Fisher Scientific Inc., Houston, TX, USA). Supernatant from each well was collected to measure LDH activity as an indication of extracellular LDH leakage. The LDH leakage ratio was expressed as extracellular LDH leakage/total LDH × 100%; total LDH was equal to extracellular LDH leakage + intracellular LDH (Koh and Choi, 1987).

Real-time reserve transcription-polymerase chain reaction (RT-PCR)
At 24 hours after the administration of Aβ25-35 expression of...
PSD-95 mRNA was determined by real-time RT-PCR. Total RNA was extracted by TRIzol agent (TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, Liaoning Province, China) and purified with RNeasy Mini kit (TaKaRa Biotechnology (Dalian) Co., Ltd.). The primers of β-actin and PSD95 were obtained from TaKaRa Biotechnology (Dalian) Co., Ltd. The nucleotide sequences of the primers for β-actin (NM031144) were, forward, 5'-GGA GAT TAC TTC GCT GGC TCT TA-3', and reverse, 5'-GAC TCA TGG TAC TCC TGC TTG CTG-3'; and for PSD95 (NM019621.1) were, forward, 5'-ACT GCA TCC TTG CGA AGC AAC-3', and reverse, 5'-CGT CAA TGA CAT GAA GGA CAT CC-3'. Total RNA was reverse-transcribed with MuLV reverse transcriptase and Oligo-dT primers. The SYBR green PCR Master Mix (Applied Biosystems, Cheshire, UK) was used for real-time PCR analysis. The relative differences in expression among groups were expressed using cycle time (Ct) values. The Ct values for the gene of interest were first normalized with β-actin in the same sample, and then the relative differences between the control and each of the other groups were calculated and expressed as a relative increase, setting the control as 100%.

**Immunocytochemical staining**

Cortical neuronal cells were fixed with 4% paraformaldehyde for 1 hour. After fixation, cells were incubated with a rabbit monoclonal anti-SYP antibody (1:125; cat. on. ab32127, Abcam, Cambridge, UK) for 1 hour at 37°C. The primary antibodies were diluted in 0.1 M phosphate buffered saline (PBS) containing 0.5% bovine serum albumin and 0.5% Triton X-100. The secondary antibodies used were Texas Red donkey anti-rabbit IgG antibody (cat. on. P0179, 1:1,000; Beyotime Institute of Biotechnology, Nanjing, China) for 30 minutes at 37°C. The fluorescence was observed using a light microscope with the appropriate fluorescence filters. The integrated optical density of active SY-P-positive expression was determined using the Imagepro-Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) in five fields in each group.

**Western blot assay**

Cortical neuronal cells were washed with cold PBS, and lysed with radioimmune precipitation lysis buffer consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 1% Triton X-100, 1% sodium deoxycholate, 1 mM Na2VO4, 1 mM phenylmethylsulfonyl fluoride, and a freshly-added protease inhibitor cocktail. The cytosols were prepared by centrifugation at 12,000 × g for 30 minutes at 4°C. Protein concentrations were quantified using the bicinchoninic acid assay (Sigma-Aldrich, St. Louis, MO, USA) (Leinenga and Götz, 2015). Equal amounts of total protein (15–30 μg) were subjected to electrophoresis on 5–12% Bis-Tris NuPAGE gels (Invitrogen), followed by electrophoretic transfer onto polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk for 1 hour, then incubated with the primary antibodies, mouse monoclonal anti-PSD95 (1:100; Abcam), rabbit polyclonal anti-β-actin (1:1,000; Beyotime Institute of Biotechnology), or rabbit monoclonal anti-SYP (1:10,000; Abcam) overnight at 4°C. After warming, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, goat anti-mouse IgG and goat anti-rabbit IgG (1:1,000; Beyotime Institute of Biotechnology) for 1 hour at room temperature. The immunoblots were visualized with enhanced chemiluminescence substrate. Images were captured using a ChemiXR4 system (Bio-Rad Laboratories, Inc., Richmond, CA, USA). The bands were scanned and analyzed using optical density values with background subtraction and normalized to β-actin using Quantity One software v4.52 (Bio-Rad Laboratories, Inc., Richmond, CA, USA).

**Statistical analysis**

All data are presented as the mean ± SD. Statistical significance was determined using analysis of variance with the Prism 5 software (GraphPad; GraphPad Software, Inc., CA, USA) followed by Dunnett's t-test. P < 0.05 was considered statistically significant.

**Results**

**Morphological change of Ab25-35-induced cortical neurons after DNLA intervention**

All the cortical neurons were prepared from 1-day-old rat pups using enzymatic digestion. The neurons were verified by morphology and staining with anti-NSE antibody (Figure 1). Populations of neurons typically with purity greater than 85% were used in the experiments. The cortical neurons were cultured continuously for 7 days. As shown in Figure 1, these cells displayed good refractive and stereoscopic properties of neuron cell body, as well as complete synaptic structures. The 7-day-old neurons were then used for drug treatments to study the protective effects of DNLA.

Ultrastructural examination of cortical neurons showed that in the control group, cell membrane and cell organelles were complete, and chromatin was distributed homogeneously (Figure 2A). After Ab25-35 treatment, chromatin was condensed and clumped, and mitochondria were swollen and vacuolar as shown in Figure 2B. Pre-treatment with DNLA significantly reduced the levels of neuronal damage compared with those of the Ab25-35 treated group. As shown in Figure 2C–E, the neurons pretreated with DNLA displayed clearer membrane structures of mitochondria and rough endoplasmic reticulum than the cells from Ab25-35 treated group (Figure 2C–E).

**DNA leakage on leakage of LDH of Ab25-35-induced cortical neurons**

Exposure of cultured neurons to Ab25-35 produced a significant increase in neuronal injury compared to the control group, as evidenced by an increase in LDH leakage (P < 0.01). DNLA treatment markedly attenuated LDH leakage (P < 0.05), indicating a protective role of DNLA against Ab25-35-induced neuronal damage (Figure 3).

**DNA leakage effects on expression of PSD-95 mRNA in Ab25-35-induced cortical neurons**

To understand the molecular events linking to the DNLA...
mediated neuroprotective mechanism, we investigated the expression level of PSD-95, a major scaffolding protein in ensuring the integrity of the neuronal synapses and receptors participating in information flow (Chen et al., 2015). As shown in Figure 4, Aβ25–35 significantly reduced the expression of PSD-95 mRNA by approximately 50% compared with the control group. However, in the DNLA (0.25 mg/L) pretreated neurons, the expression levels of PSD-95 mRNA were significantly higher compared to the expression of PSD-95 after Aβ25–35 treatment alone (P < 0.05) (Figure 4).

DNLA effects on the expression of SYP and PSD-95 protein in Aβ25–35–induced cortex neuron cells
As shown in Figure 5A–C, Aβ25–35 challenge resulted in severe synaptic damage, and loss of synaptic connections. Immunoreactivity of SYP-positive expression decreased significantly compared with that in the control group (P < 0.01). These morphological changes were significantly attenuated by the pretreatment with DNLA in a dose-dependent manner. The immunoreactivity of positive expression increased significantly in DNLA groups compared with Aβ25–35 treated group (DNLA 0.25 mg/L group, P < 0.05; DNLA 2.5 mg/L group, P < 0.01). We further determined the protein expression of SYP and PSD-95 by western blot assay. As shown in Figure 5C, Aβ25–35 treatment decreased significantly the expression of SYP (P < 0.05) and PSD-95 (P < 0.01) proteins compared with the control group, while the expression levels of both proteins were significantly prevented when cells were pretreated with DNLA. Clearly, our data demonstrated that DNLA possesses a neuroprotective effect on cortical neurons.

Growing attention in the recent decade has been paid to synaptic dysfunction due to the synaptotoxic effects of Aβ. Neuropathological studies in humans have supported the findings that synaptic loss occurs at an earlier stage of AD than gross neuronal injury (Götz and Ittner, 2008; Marcello et al., 2012). In addition, there was a positive correlation between the degree of synaptic loss and synaptic dysfunction and the cognitive impairment in AD patients (Clare et al., 2010). All these suggest the important roles of synaptic structure and function in the development of AD.

SYP is one of the major glycosylated protein found in presynaptic vesicles. It has been considered as a specific marker for the nerve terminals (Canas et al., 2014). In addition, SYP is a marker for the functionality of the synapse. It is also important for the release of LDH caused by Aβ25–35 in cortical neurons. Furthermore, we showed that pretreatment of DNLA significantly blocked the decrease in the expression of both synaptic proteins SYP and PSD-95. These molecular changes may be central to the DNLA-mediated synaptic protection.

Discussion
In this study, we clearly demonstrated that DNLA has a neuroprotection role against Aβ25–35–induced cytotoxicity in primary cultured rat cerebral cortical neurons. DNLA significantly attenuated the damage to morphological structures and inhibited the release of LDH caused by Aβ25–35 in cortical neurons. Furthermore, we showed that pretreatment of DNLA significantly blocked the decrease in the expression of both synaptic proteins SYP and PSD-95. These molecular changes may be central to the DNLA-mediated synaptic protection.

A wealth of evidence indicates that accumulation of excessive intraneuronal Aβ initiates a complex cascade of neurotoxic alterations (Gaspar et al., 2010). Aβ, a major component of diffuse plaques in AD brain, results in the production of neurotoxins and causes synaptotoxic effects, leading ultimately to degeneration of neurites and neuronal cell bodies. These pathological changes result in cognitive impairment and the development of dementia (Pike et al., 2011). Aβ is not only the origin but also the common pathway in the occurrence and development of AD. Inhibition of these Aβ–induced toxic effects has been a key area in the study of the pathological progression and in the exploration of effective treatments for AD. In the present study, we observed that Aβ25–35–elicited neuronal toxicity, including an increase in the leakage of LDH and disruption of neuronal cell organelles. However, these cytotoxic effects were significantly prevented when cells were pretreated with DNLA. Clearly, our data demonstrated that DNLA possesses a neuroprotective effect on cortical neurons.

Figure 1 Identification of cortical neurons (× 400).
(A) Immunocytochemical staining of cortical neurons by anti-neuron-specific enolase antibody. Cortical neurons (arrows) cultured for 7 days typically had a purity level greater than 85%. (B) Primary culture of rat neurons for 7 days: Cells grew well, had interlaced distribution with clear borders and good cell morphology when observed by inverted phase contrast microscope. Arrows in B indicate normal neurons.

Figure 2 Effects of DNLA administration on the ultrastructural changes induced by Aβ25–35 in rat primary cultured neurons detected by transmission electron microscope.
(A) Control group: Cell membrane and cell organelles were complete, and chromatin was distributed homogeneously. (B) Aβ25–35 exposed group: chromatin was condensed and clumped, and mitochondria were swollen and vacuolar. (C–E) Pretreatment with DNLA (0.025, 0.25, 2.5 mg/L) groups, 24 hours before Aβ25–35 exposure: structures of the mitochondria and rough endoplasmic reticulum are clearly visible. Scale bars: 1.0 μm. Arrows indicate examples of normal neurons. DNLA: Dendrobium nobile Lindl. alkaloids; Aβ25–35: amyloid-beta (25–35).
involved in the process of exocytotic release of neurotransmitter and membrane recycling in nerve terminals (Vukic et al., 2009; Gordon et al., 2011). In this study, we examined both the density and distribution of SYP in order to reflect the synaptic density and distribution of the synapse.

PSD-95, a postsynaptic scaffolding protein, is distributed evenly throughout the PSD which is a complex, disk-shaped structure attached to the postsynaptic membrane at synapses (Chen et al., 2011). PSD-95 binds to the glutamate receptors and to other components of the PSD, including Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), Shank, and actin (Chen et al., 2011). Several studies have indicated the involvement of PSD-95 in the trafficking of α-aminoo-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors, and gating of N-methyl-D-aspartate receptors (Opazo et al., 2012; Genevieve et al., 2014). Trafficking of receptors at the PSD results in changes in synaptic transmission and structure, and these changes are responsible for the formation, function, and plasticity of spine synapses.

In this study, the results of immunocytochemistry and

Figure 3 DNLA effects on Aβ25-35-induced leakage of LDH in rat primary cultured neurons.

Rat primary cultured neurons were pretreated with DNLA for 24 hours before the addition of Aβ25-35. 24 hours later, the leakage of LDH was determined by biochemical instrument. Results are expressed as the mean ± SD from three independent experiments, and analyzed by analysis of variance followed by Dunnett’s t-test. ***P < 0.01, vs. control group; #P < 0.05, vs. Aβ25-35 treated group. I: Control group, without treatment; II: Aβ25-35 group, treated with 10 μM Aβ25-35 for 24 hours; III–V: Neuronal cultures pretreated with 0.025, 0.25, 2.5 mg/L DNLA, respectively, for 24 hours, followed by 10 μM Aβ25-35 administration for 24 hours. DNLA: Dendrobium nobile Lindl. alkaloids; Aβ25-35: amyloid-beta (25–35); LDH: lactate dehydrogenase.

Figure 4 Effects of DNLA and Aβ25-35 on mRNA expression of PSD-95 in rat primary cultured neurons.

Rat primary cultured neurons were pretreated with DNLA for 24 hours and then treated with Aβ25-35. The mRNA expression of PSD-95 was measured by real-time reverse transcription-polymerase chain reaction. Results are expressed as the mean ± SD from five independent experiments, and analyzed by analysis of variance followed by Dunnett’s t-test. ***P < 0.01, vs. control group; #P < 0.05, vs. Aβ25-35-treatment; II: Aβ25-35-treated group, treated with 10 μM Aβ25-35 for 24 hours; III–V: Neuronal cultures treated with 0.025, 0.25, 2.5 mg/L DNLA, respectively, for 24 hours, followed by 10 μM Aβ25-35 intoxication for 24 hours. DNLA: Dendrobium nobile Lindl. alkaloids; Aβ25-35: amyloid-beta (25–35); PSD-95: postsynaptic density-95.

Figure 5 Effects of DNLA on Aβ25-35-induced protein expression of synapse-associated proteins in rat primary cultured neurons.

Rat primary cultured neurons were pretreated with DNLA for 24 hours before 24 hours exposure to Aβ25-35. (A) Neurons were immunofluorescence stained with anti-SYP antibody (× 200). (B) The quantification of SYP expression. (C) The protein level of SYP and PSD-95 was determined by western blot assay. Optical density of SYP or PSD-95 band was normalized to that of β-actin. Results are expressed as the mean ± SD from five independent experiments, and analyzed by analysis of variance followed by Dunnett’s t-test. *P < 0.05, **P < 0.01, vs. control group; #P < 0.05, ##P < 0.01, vs. Aβ25-35-treated group. I: Control group, without treatment; II: Aβ25-35-treated group, treated with 10 μM Aβ25-35 for 24 hours; III–V: Groups treated with 0.025, 0.25, 2.5 mg/L DNLA, respectively, for 24 hours, followed by 10 μM Aβ25-35 intoxication for 24 hours. DNLA: Dendrobium nobile Lindl. alkaloids; Aβ25-35: amyloid-beta (25–35); SYP: synaptophysin; PSD-95: postsynaptic density-95.
western blot assay suggest that DNLA effectively suppressed the decrease in the level of density and expression of SYP protein caused by Aβ25-35 treatment. After treatment with DNLA, the levels of PSD-95 mRNA and protein in primary cultured neurons were enhanced. This suggests that DNLA may play a role in maintaining the integrity of pre-synaptic and post-synaptic structures, thereby improving the synaptic connection, synaptic transmission and synaptic plasticity. These molecular events associated with DNLA might play a key role in improving the information transmission of the nervous system that is involved in the mechanism of DNLA-mediated neuroprotective mechanism.

In summary, DNLA protected cortical neurons against Aβ25-35-mediated neurotoxicity and synaptic damage. This synaptic protective effect was at least partially mediated by the reduced Aβ25-35-induced decrease in SYP and PSD-95. Thus, DNLA might possess a potential benefit for the prevention and early therapy of AD. Further studies are required to fully define the underlying molecular mechanism of DNLA elicited neuroprotection. This work provides a pharmacological basis for DNLA in prevention and treatment of AD, especially in the early stage of AD.

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Conflicts of interest: None declared.

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