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Neuroprotective effects of tenuigenin in a SH-SY5Y cell model with 6-OHDA-induced injury

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ABSTRACT

Tenuigenin, an active component of *Polygala tenuifolia* root extracts, has been shown to provide antioxidative and anti-aging effects in Alzheimer's disease, as well as to promote proliferation and differentiation of neural progenitor cells. However, the effects of tenuigenin on Parkinson's disease remain unclear. In the present study, SH-SY5Y cells were utilized to determine the effects of tenuigenin on 6-hydroxydopamine (6-OHDA)-induced injury. Results showed that 1.0×10^{-1}–10 μM tenuigenin significantly promoted cell viability and reduced cell death. In addition, tenuigenin protected mitochondrial membrane potential (MMP) against 6-OHDA damage and significantly increased glutathione and superoxide dismutase expression. At the mRNA level, tenuigenin resulted in down-regulation of caspase-3, but up-regulation of tyrosine hydroxylase expression in 6-OHDA damaged cells. These results suggested that tenuigenin provides neuroprotection to dopaminergic neurons from 6-OHDA-induced damage. The neuroprotective mechanisms might involve antioxidative effects, maintenance of mitochondrial function, and regulation of caspase-3 and tyrosine hydroxylase expression and activity. Tenuigenin could provide a novel antioxidative strategy for Parkinson's disease.

Tenuigenin (TEN), an extract from the Chinese herb *Polygala tenuifolia*, has been used to improve memory and cognitive functions in Traditional Chinese Medicine (TCM) for 2000 years. In addition, *P. tenuifolia* root extracts have been reported to exhibit neuroprotective and neuroregenerative effects [15], as well as enhance cognitive functions in elderly individuals and provide memory enhancement in healthy adults [11,18]. In a rat model of Alzheimer's disease (AD), *P. tenuifolia* root extracts ameliorate spatial cognition disorders and protect neuronal cells against *in vitro* toxins [6,20,23]. Cell culture results have shown that *P. tenuifolia* treatment decreases secretion of amyloid β-protein, which is responsible for neuronal pathogenesis and cell death in AD [8,14]. BT-11, the extract of *P. tenuifolia* Willdenow roots, has been shown to ameliorate memory impairments induced by scopolamine and stress in rats [19], as well as protect culture neuronal cells against toxins. These results suggest that root extracts of *P. tenuifolia* are active constituents and could be used for therapeutic purposes in neurodegenerative diseases, in particular AD.

Parkinson's disease (PD), the second most common neurodegenerative disorder, results from progressive loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNpc) [10]. PD clinical symptoms, such as resting tremors, postural instability, rigidity, and bradykinesia, significantly increase at approximately 80% loss of DA neurons [3,4]. PD pathogenesis remains poorly understood, although it is thought that oxidative stress and mitochondrial dysfunction play an important role [4,16]. The oxidative metabolite of dopamine, 6-hydroxydopamine (6-OHDA), acts as a neurotoxin and has been used to establish experimental animal models of PD [1,17]. The metabolite 6-OHDA induces cytotoxicity in various cell types [21]. The downstream mechanisms of these toxic products could be involved in increased mitochondrial outer membrane permeability (MOMP), which would lead to release of cytochrome c and other pro-apoptotic mitochondrial proteins that activate downstream effectors, such as caspase 3 [5,7].

A previous study from our laboratory explored the use of novel drugs for Parkinson's disease [12,13]. The *P. tenuifolia* root has been used as a traditional herbal medicine for the treatment of patients with insomnia, neurosis, and dementia [15]. TEN is an active, lipophilic ingredient in *P. tenuifolia* (molecular formula: $C_{30}H_{45}ClO_6$; relative molecular mass: 537) (Supplemental Fig. 1). TEN exhibits a variety of biological activities, including antioxidative and anti-aging effects. However, it remains to be shown whether TEN provides neuroprotection in PD. The present study hypothesized that tenuigenin could provide neuroprotective effects for PD.

Human, neuronal, 6-OHDA-injured SH-SY5Y cells served as a PD cell model and were used to explore the effects of TEN on PD. SH-SY5Y cells were cultured in Dulbecco's-modified Eagle medium (Invitrogen, San Diego, CA) supplemented with 10% FBS

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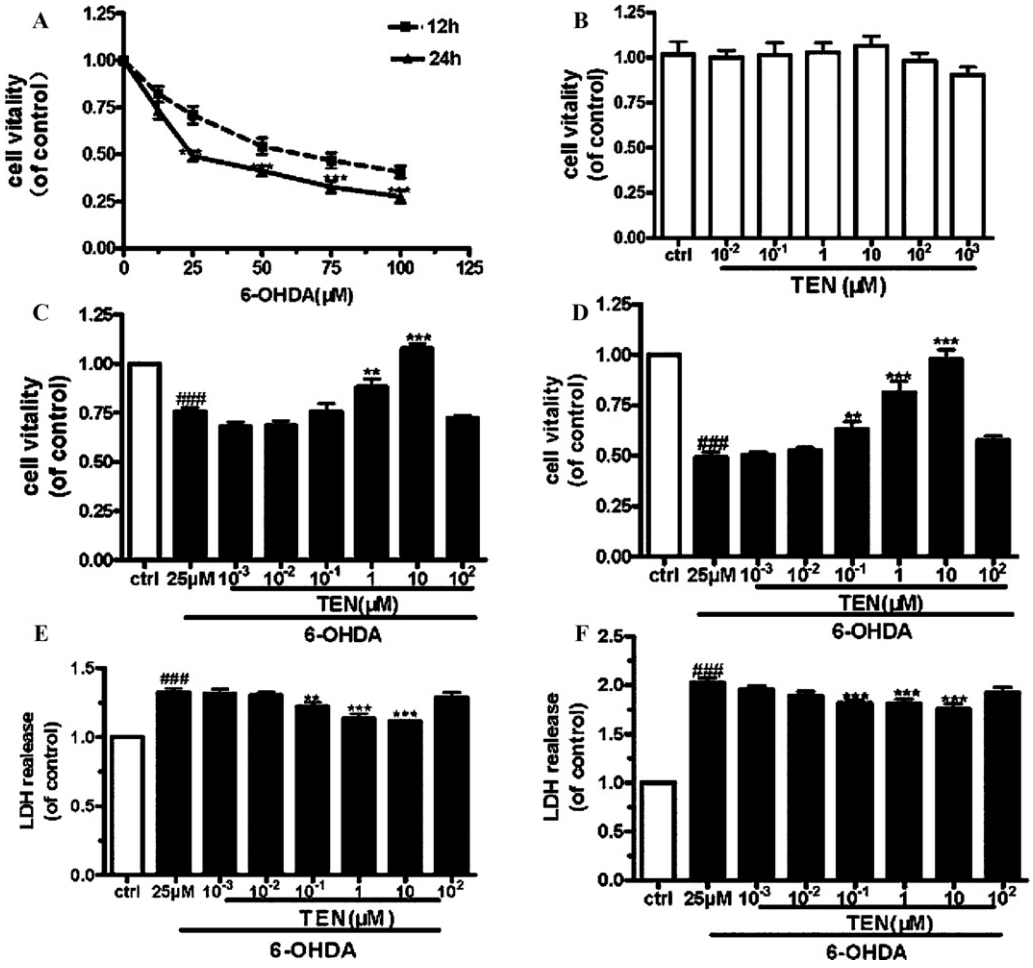

Fig. 1. Decreased survival of dopaminergic neuronal cells (SH-SY5Y) following 6-OHDA treatment. SH-SY5Y cells were pre-treated with different concentrations of tenuigenin for 1 h, followed by exposure to 25 μM 6-OHDA for 24 h. Cell viability assay was performed with MTS. Different tenuigenin doses (1×10^{-2}–10^3 μM) do not alter SH-SY5Y cell viability at 24 h; however, 1×10^{-1}–10 μM tenuigenin pre-treatment increases cell viability following 6-OHDA exposure at 12 or 24 h. In addition, 1×10^{-1}–10 μM tenuigenin pre-treatment decreases LDH release. (A) SH-SY5Y cells treated with 12.5, 25, 50, 75, or 100 μM 6-OHDA for 12–24 h. (B) Effects of tenuigenin (1×10^{-2}–10^3 μM) on SH-SY5Y cells at 24 h. (C and D) Effects of tenuigenin on SH-SY5Y cell and 6-OHDA damage in SH-SY5Y cells. SH-SY5Y cells were pre-treated with different concentrations of tenuigenin for 1 h followed by exposure to 25 μM 6-OHDA for 12 or 24 h (E and F). Effect of tenuigenin on LDH in SH-SY5Y cells damaged by 6-OHDA at 12 or 24 h. These values represent mean ± SEM in triplicate experiments ($n = 3$) (**, $^{\#\#}P < 0.01$;***, $^{\#\#\#}P < 0.001$; *vs.* 6-OHDA group, $^{\#}$*vs.* control).

(Hiclone, Logan, Utah, USA), streptomycin (10 μg/ml), and penicillin (10 U/ml) at 37 °C in a 5% CO_2 humidified incubator. The neurotoxin 6-OHDA (Sigma, St. Louis, MO) was dissolved in PBS, and 6-OHDA stocks (50 mM, 1000×) were made in ascorbic acid (0.01%) and added to culture medium to achieve the required final concentration (25 μM). Subconfluent cells were exposed to 6-OHDA for 12 or 24 h, and cell viabilities were determined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega). Cell death was determined using a commercial lactate dehydrogenase (LDH) assay kit (Promega, Madison, WI, USA) and conditioned media samples were obtained from control and 6-OHDA-treated cells. Selegiline (SEL) (L-deprenyl, Eldepryl) (Tocris Bioscience, St. Louis, MO, USA), a selective inhibitor of monoamine oxidase B commonly used to treat PD [24], was selected as a positive treatment control for the study. The concentration of the SEL was 10 μM.

The MTS assay (CellTiter96 AQ, Promega) was utilized to test cell viability. SH-SY5Y cells were seeded in 96-well plates at a density of 5×10^3 cells/well for 24 h prior to experimentation. Subsequently, SH-SY5Y cells were induced by different doses of 6-OHDA (12.5–100 μM) in 5% fetal bovine serum/cell culture medium for 24 h. However, control cells were treated with saline instead. TEN (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) was administered at a dose of (10^{-3}, 10^{-2}–10^2 μM) at 1 h prior to the addition of 6-OHDA into SH-SY5Y cell cultures for 24 h. Supernatants from each well were separately harvested and used to determine LDH activity using the spectrophotometrical method and LDH analysis kits (Promega). Cell cytotoxicity was measured by LDH at 12 and 24 h after 6-OHDA treatment. Cell culture medium was removed and cells were washed with phosphate-buffered saline (PBS), followed by the addition of 10 μL MTS in 100 μL complete serum-free medium into each well. After incubation at 37 °C for 2 h, the absorbance value at 490 nm was measured on a spectrophotometer (BioRad, Hercules, CA, USA) and cell viability was converted and expressed as the percentage of control. Experiments were performed in triplicate.

Total SOD levels in treated and untreated cell extracts were measured using the OxiSelect™ Superoxide Dismutase Activity Assay (Cell Biolabs, San Diego, CA, USA). SH-SY5Y cells were seeded into 6-well plates at a density of 1×10^6 cells/well according to the assay protocol. SOD activity was measured at 12 or 24 h, and total GSH levels were measured using a luminescence assay test kit (GSH-GloTM Glutathione Assay Kit; Promega). GSH activity was measured in 96-well plates at 12 or 24 h, and activity was reported as a luminescence light unit.