

Neuroprotective effects of phenylethanoid glycosides in an *in vitro* model of Alzheimer's disease

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Received January 8, 2016; Accepted January 6, 2017

DOI: 10.3892/etm.2017.4254

Abstract. The present study aimed to investigate the neuroprotective effects of phenylethanol glycosides (PhGs) on H₂O₂- and β -amyloid peptide (A β)₁₋₄₂-induced injury of PC12 cells as an *in vitro* model of Alzheimer's disease (AD). The optimal induction conditions were established through screening of various incubation times and concentrations. PC12 cells were treated with 0.5 μ M A β ₁₋₄₂ and H₂O₂ in the presence of PhGs for 24 h and the cell viability was then evaluated by an MTT assay; lactate dehydrogenase (LDH) release and malondialdehyde (MDA) content were also measured. The optimal conditions for establishing the AD model were the treatment of PC12 cells with 0.5 μ M A β ₁₋₄₂ for 48 h, or with 25 μ M H₂O₂ dissolved in DMEM with PBS. PhGs at concentrations of 5, 25 and 50 μ g/ml increased the viability and decreased LDH and MDA release by PC12 cells injured with A β ₁₋₄₂ or H₂O₂. In conclusion, the model of A β ₁₋₄₂- and H₂O₂-induced PC12 cell injury was successfully established. PhGs were shown to have a significant neuroprotective effect against A β ₁₋₄₂- or H₂O₂-induced cell injury.

Introduction

Alzheimer's disease (AD), a neurodegenerative disorder with the clinical characteristics of progressive memory loss and cognitive function impairment (1), is the most common cause of dementia worldwide (2). The financial costs are immense due to the prevalence of AD (3). Hence, it is urgent to develop appropriate means for the management and prevention of AD.

The pathogenesis of AD is closely associated with the accumulation of neurofibrillary tangles and senile plaques (SPs) in affected brain regions (4,5). β -amyloid peptide (A β), the major component of SPs, has been reported to have a causative role in the progression of AD as has a toxic effect on neuronal cells (6). A β fragments, including A β ₁₋₄₀, A β ₂₅₋₃₅ and A β ₁₋₄₂, have been generated through the split of amyloid precursor protein (7). The neurotoxicity of A β ₁₋₄₂ was found to be significantly higher than that of A β ₂₅₋₃₅ and A β ₁₋₄₀, and A β ₁₋₄₂ is able to induce an AD model (1,8-10). Oxidative stress may be involved in the pathogenesis of AD and is the major mechanism underlying A β -induced neurotoxicity (11-13). Several studies suggested that A β ₁₋₄₂ caused intracellular accumulation of reactive oxygen species (ROS), leading to lipid and protein oxidation, DNA damage and activation of cell cycle checkpoint signaling (1,14,15). Excessive amounts of H₂O₂ may lead to oxidative damage and induce apoptosis of PC12 cells (16). Therefore, targeting of oxidative stress may be a promising approach for the development of therapeutic strategies for inhibiting A β -induced neurotoxicity in AD.

Herba (H.) Cistanche, a Chinese herbal medicine commonly used in mainland China for nourishing the kidneys and replenishing essence and blood, has been used to treat memory loss and senile constipation (17). Phenylethanoid glycoside (PhG), one of the major constituents in *H. Cistanche*, improves the impairment of neuronal apoptosis caused by A β ₂₅₋₃₅ via its anti-oxidant effects (18,19). A previous study identified five major components from total PhGs, namely acteoside, 2'-acetylacteoside, echinacoside, cistanosides and isoacteoside (20). Among these components, acteoside and echinacoside have been reported to have neuroprotective effects on A β ₂₅₋₃₅- or H₂O₂-induced neurotoxicity (21-23). For instance, Wu *et al* (24) suggested that acteoside and echinacoside ameliorated cognitive dysfunction caused by A β ₁₋₄₂. The present study aimed to investigate the protective effects of PhGs in an *in vitro* rat cell model of AD.

Materials and methods

Preparation of PhGs. Total PhGs were extracted from *H. Cistanche* as previously described (25). The air-dried stem of *H. Cistanche* was powdered and extracted by percolation with 80% EtOH. The percolate was evaporated under reduced

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Key words: phenylethanol glycosides, PC12 cells, H₂O₂, β -amyloid peptide₁₋₄₂, neuroprotection

pressure, followed by re-suspension in an appropriate amount of H_2O_2 (100 $\mu\text{mol/l}$). The mixture was isolated on an SP-825 macroporous resin column (Mitsubishi Chemical, Tokyo, Japan) and eluted with 0, 30, 50, 70 and 90% EtOH in water. To obtain the PhG-rich fraction, the 30-50% EtOH eluents were concentrated and dried under reduced pressure. Ultraviolet (UV) spectrophotometry was performed to determine the total PhGs. The content of echinacoside and acteoside was determined by high-pressure liquid chromatography according to a previous protocol (26). A Hypersil ODS-2 column (4.6x250 mm, 5 μm ; Dalian Elite Analytical Instruments, Co., Ltd., Dalian, China) was used and maintained at room temperature. The mobile phases were methyl cyanides and water containing 0.4% phosphoric acid (v/v; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The flow rate was 0.75 ml/min and the wavelength was set to 333 nm.

Cell culture and drug treatment. The PC12 rat pheochromocytoma cell line was provided by Dr He Chunhui, the Medical School, Xinjiang Medical University (Urumqi, China). Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Sangon Biotech Co. Ltd., Shanghai, China), 100 U/ml penicillin and 100 U/ml streptomycin in an incubator at 37°C containing 5% CO_2 and 95% air. Upon reaching 80% confluence, the cells were treated with 0.25% trypsin and passaged.

In order to eliminate the interference of the drug itself on the growth of PC12 cells, a toxicity experiment was performed. In brief, PC12 cells (3×10^4 cells/ml) were seeded in 96-well plates at 100 μl /well and incubated at 37°C overnight. After discarding the supernatant, 200 μl complete DMEM was added in the blank group, while cells in the intervention groups were treated by PhGs at various doses (5, 25, 50, 75, 100, 125, 150, 175 and 200 $\mu\text{g/ml}$). Following incubation of the cells at 37°C for 48 h, 20 μl MTT solution (Sigma-Aldrich; Merck KGaA) was added to each well. After incubation for 4 h, the supernatant was discarded and 150 μl dimethylsulfoxide was added per well, followed by agitation for 10 min. The optical density (OD) values at 490 nm were detected using an ELISA plate reader.

$\text{A}\beta_{1-42}$ -induced PC12 cell injury. $\text{A}\beta_{1-42}$ peptide purchased from Bioss Biotech (Beijing, China) was dissolved in water (100 $\mu\text{g/ml}$). Subsequently, the mixture was incubated at 37°C for 4 days and stored at 4°C prior to use.

PC12 cells were seeded in 96-well plates (3×10^4 cells in 100 μl per well). After culture for 24 h for adherence, 50 μl $\text{A}\beta_{1-42}$ at various final concentrations (0, 0.25, 0.5, 1, 1.5 or 2 μM) dissolved in serum-free DMEM was added, followed by incubation for 24, 48, 72 or 96 h. Cell viability was evaluated by an MTT assay. The optimal $\text{A}\beta_{1-42}$ concentration was 0.5 determined to be μM .

PC12 cells (3×10^4 cells per well) were treated with various doses of PhGs (0, 0.5, 5, 25 or 50 $\mu\text{g/ml}$) in the presence of 0.5 μM $\text{A}\beta_{1-42}$ for 24 h. Cell viability was evaluated by an MTT assay.

H_2O_2 -induced PC12 cell injury. PC12 cells were plated seeded in 96-well plates (3×10^4 cells in 100 μl per well). After culture

for 24 h for adherence, 100 μl H_2O_2 at various final concentrations (0, 25, 50, 100, 200, 300, 400 and 500 μM) dissolved in DMEM with or without PBS (0.01 mol/l) was added, followed by incubation for 24 h. Cell viability was evaluated by an MTT assay. The optimal H_2O_2 concentration and solvent was determined to establish the *in vitro* model of AD.

PC12 cells (3×10^4 cells per well) were treated with various doses of PhGs (0, 0.5, 5, 25 and 50 μM). After culture for 24 h for adherence, PC12 cells were treated with 100 μl H_2O_2 dissolved in DMEM with PBS in the presence of PhGs for 24 h. The cell viability was evaluated by an MTT assay.

Lactate dehydrogenase (LDH) release assay. Cell injury was assessed through measuring the LDH activity in the supernatant of PC12 cells using an LDH kit according to the manufacturer's protocol (cat. no. 20150604; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). In brief, double-distilled H_2O , 0.2 $\mu\text{mol/ml}$ pyruvic acid, matrix buffer and coenzyme I buffer were added in sequence at 48 h after drug treatment. After incubation at 37°C for 15 min, 2,4-dinitro-phenylhydrazine was added. Subsequently, 250 μl of a 0.4 M NaOH solution was added to each well. The supernatant was collected after incubation for 30 min at room temperature. The absorbance at 450 nm was then measured with a microplate reader.

Measurement of malondialdehyde (MDA). MDA was measured in the supernatant of PC12 cells using commercial kit (cat. no. 20150604; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol. In brief, dehydrated alcohol and other reagents were added in order, followed by incubation in a water bath at 95°C for 40 min. The mixture was centrifuged at $1,006 \times g$ and 25°C for 10 min after cooling. The supernatant was then used to determine the MDA content. Absorbance was subsequently measured with a microplate reader at 532 nm.

Assessment of protective effects of echinacoside and acteoside against AD *in vitro*. PC12 cells were seeded at a density of 3×10^4 cells/well in 96-well plates (100 μl /well). Cells were incubated with drugs including echinacoside (cat. no. 111670-200503; National Institutes for Food and Drug Control, Beijing, China) and acteoside (cat. no. 111530-200505; National Institutes for Food and Drug Control) at various concentrations (0.5, 25 and 50 $\mu\text{g/ml}$). Subsequently, the cells were treated with $\text{A}\beta_{1-42}$ or H_2O_2 for 24 h and the cell viability was measured by an MTT assay.

Statistical analysis. Values are expressed as the mean $\pm$ standard deviation. Student's t-test was used for inter-group comparisons. Statistical analyses were performed using SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Quantification of PhGs from *H. Cistanches*. The UV spectra of the PhGs extracted as well as standard solutions of echinacoside and acteoside were recorded, and the results showed that the UV spectra were consistent (Fig. 1). UV spectrophotometry

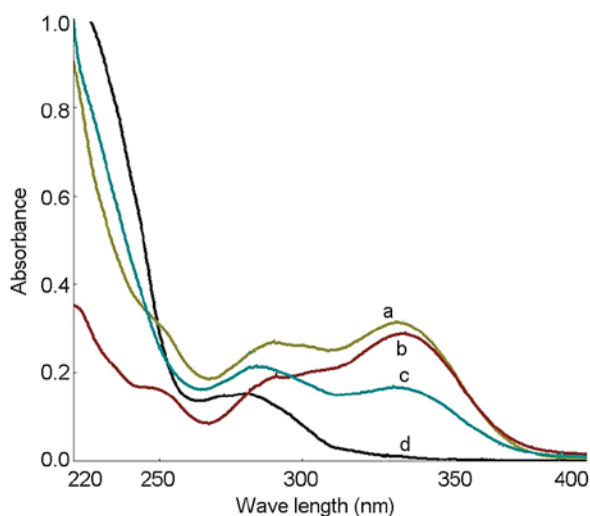


Figure 1. Ultraviolet spectrum of (a) echinacoside, (b) acteoside (c) phenylethanoid glycosides extracted in the present study, and (d) blank control.

showed that the PhG content was 87.6%. The HPLC results showed that the contents of echinacoside and acteoside were 37.7 and 17.8%, respectively (Fig. 2).

Determination of the ideal PhG concentration. Compared with the blank group, PhG at 75, 100, 125, 150, 175 and 200 $\mu\text{g/ml}$ had a significant inhibitory effect on PC12 cells ($P < 0.05$), while PhG at 5, 25 and 50 $\mu\text{g/ml}$ showed low toxicity on PC12 cells, and the cell viability was $>80\%$ (Fig. 3). Thus, PhGs at the concentration of 5, 25 and 50 $\mu\text{g/ml}$ was used for treating PC12 cells in subsequent experiments due to not affecting the cell viability.

$A\beta_{1-42}$ -induced PC12 cell injury. Compared with that in the control group, the cell viability in the 0.5 μM $A\beta_{1-42}$ injury group was 63% ($P < 0.05$). The cell viability was decreased by $A\beta_{1-42}$ in a concentration-dependent manner, and the viability was $<50\%$ in the 1, 1.5 and 2 μM $A\beta_{1-42}$ injury groups (Fig. 4). Thus, treatment with 0.5 μM $A\beta_{1-42}$ for 48 h was determined to be the optimal condition for establishing the *in vitro* AD model.

The activity of PC12 cells treated with 0.5 μM $A\beta_{1-42}$ in the presence of safe doses of PhGs (5, 25 and 50 $\mu\text{g/ml}$) for 24 h was also determined. Compared with the model group ($P < 0.01$), PhGs showed a significant neuroprotective effect on PC12 cells. The cell viability was rescued by PhGs in a dose-dependent manner (Fig. 5).

H_2O_2 -induced PC12 cell injury. The viability of PC12 cells treated with 25 μM H_2O_2 dissolved in DMEM with PBS was 56.43%. The viability of PC12 cells treated with 200 μM H_2O_2 dissolved in DMEM without PBS was 71.64% (Table I). Thus, PC12 cells treated with 25 μM H_2O_2 dissolved in DMEM with PBS was the selected as the optimal condition for establishing the AD model.

Compared with the control group, the cell viability in the model group was 48.8% ($P < 0.05$). Compared with the model group, PhGs had a significant neuroprotective effect on PC12 cells. The cell viability was dose-dependently increased by

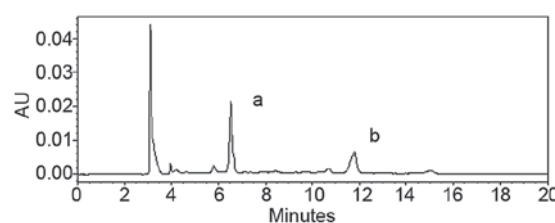


Figure 2. High-performance liquid chromatography spectrum showing (a) echinacoside and (b) acteoside.

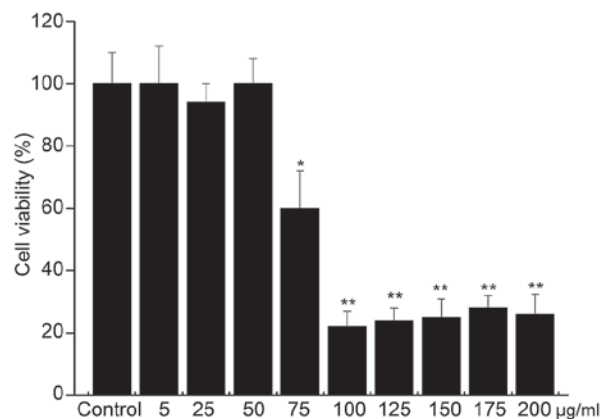


Figure 3. Dose screening of phenylethanoid glycosides to determine an optimal concentration to not affect cell viability. * $P < 0.05$, vs. control; ** $P < 0.05$, vs. control.

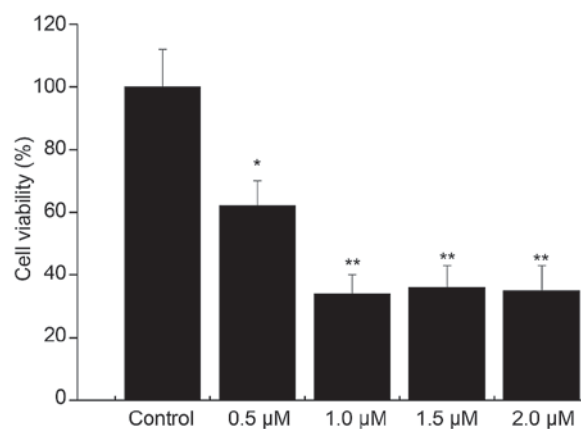


Figure 4. Screening of $A\beta_{1-42}$ damage conditions. * $P < 0.05$, vs. control; ** $P < 0.05$, vs. control.

PhGs, and the viability of PC12 cells treated with PhGs at concentrations of 5, 25 and 50 $\mu\text{g/ml}$ was 54, 57 and 64%, respectively (Table II).

PhGs inhibit injury-induced LDH release by PC12 cells. Compared with the control group, the LDH content of the supernatant of injured PC12 cells was increased, which was inhibited by PhGs in a concentration-dependent manner. This result indicated that PhGs have a significant neuroprotective effect on PC12 cells (Fig. 6).

Table I. Cell viability (%) after H₂O₂ treatment.

H ₂ O ₂ concentration (μ M)	Solvent	
	DMEM+PBS	DMEM
0	100	100
25	56.43 ^a	107.61
50	54.81 ^a	108.13
100	52.79 ^a	105.01
200	46.30 ^a	71.64 ^a
300	53.90 ^a	60.06 ^a
400	44.28 ^a	61.00 ^a
500	43.68	60.17 ^a

^aP<0.05 vs. control group. PhGs, phenylethanoid glycosides; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.

Table II. Cell viability after drug interference.

Group	Cell viability (%)
Control	100
Model	48.83 ^a
PhG 5 μ g/ml	53.94 ^b
PhG 25 μ g/ml	57.39 ^b
PhG 50 μ g/ml	64.00 ^c

^aP<0.05, vs. control group; ^bP<0.05, vs. model group; ^cP<0.05, vs. model group. PhGs, phenylethanoid glycosides.

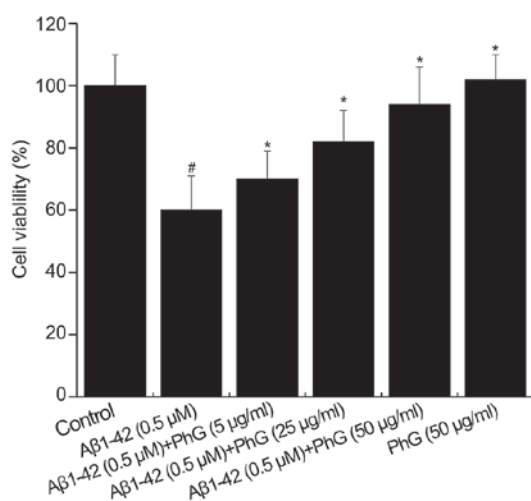


Figure 5. Cell viability of PC12 cells treated with A β_{1-42} and PhGs. [#]P<0.05, vs. control group; ^{*}P<0.05, vs. model group. A β , β -amyloid peptide; PhGs, phenylethanoid glycosides.

PhGs inhibit injury-induced MDA production by PC12 cells. Compared with the control group, the MDA content in the supernatant of injured PC12 cells was increased, which was

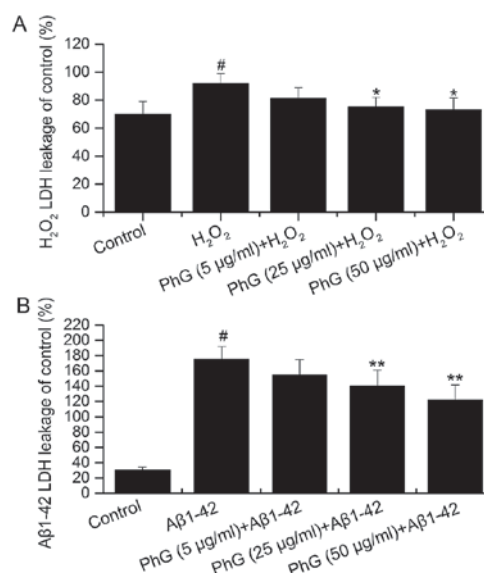


Figure 6. LDH release after (A) H₂O₂ interference and (B) A β_{1-42} interference in an *in vitro* model of Alzheimer's disease. [#]P<0.05, vs. control group; ^{*}P<0.05, vs. model group; ^{**}P<0.05, vs. model group. LDH, lactate dehydrogenase; A β , β -amyloid peptide; PhGs, phenylethanoid glycosides.

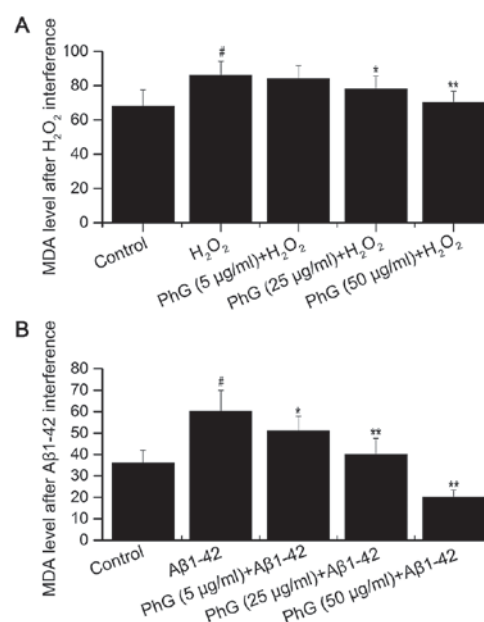


Figure 7. Determination of MDA after (A) H₂O₂ interference and (B) A β_{1-42} interference in an *in vitro* model of Alzheimer's disease. [#]P<0.05, vs. control group; ^{*}P<0.05, vs. model group; ^{**}P<0.05, vs. model group. MDA, malondialdehyde; A β , β -amyloid peptide; PhGs, phenylethanoid glycosides.

inhibited by PhGs in a concentration-dependent manner. This result indicated that PhGs have a significant neuroprotective effect on PC12 cells (Fig. 7).

PhG and its components echinacoside and acteoside rescue the viability of injured PC12 cells. Compared with the model group, treatment with acteoside significantly increased the viability of A β_{1-42} -injured PC12 cells in a dose dependent manner. PhGs and echinacoside also significantly increased the viability of A β_{1-42} -injured PC12 cells at all concentrations tested (Fig. 8A).

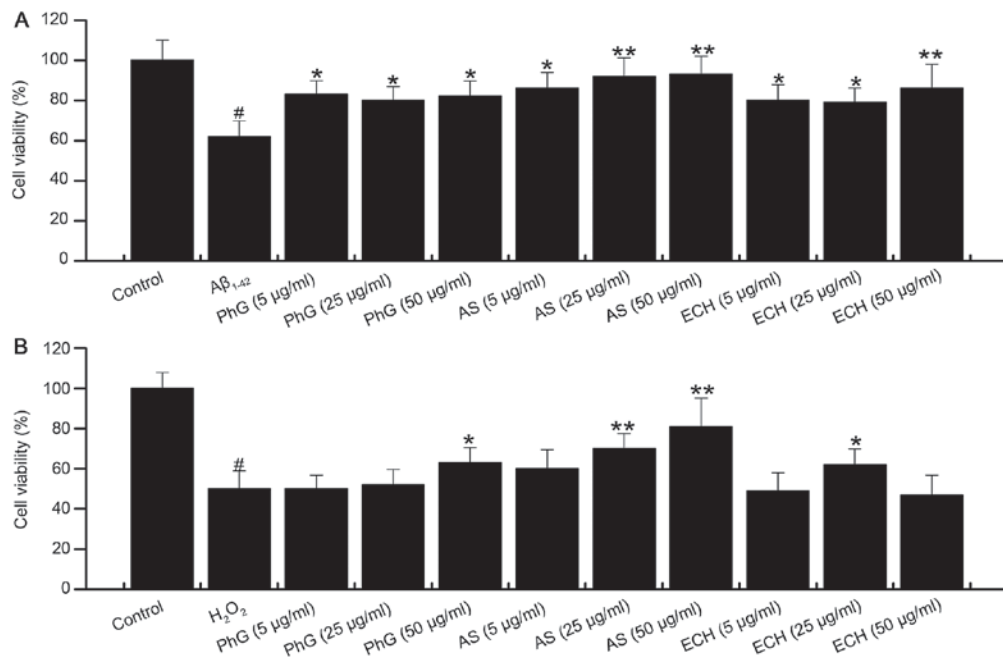


Figure 8. Protective effects of PhGs, ECH and AS on the cells treated with (A) Aβ₁₋₄₂ and (B) H₂O₂. #P<0.05, vs. control group; *P<0.05, vs. model group; **P<0.05, vs. model group. Aβ, α -amyloid peptide; PhGs, phenylethanoid glycosides; ECH, echinacoside; AS, acteoside.

Compared with the model group, acteoside significantly increased the viability of PC12 cells treated with H₂O₂. PhGs also increased the viability of PC12 cells treated with H₂O₂, while the effect was not significant at concentrations of 5 and 25 μg/ml (Fig. 8B). In addition, echinacoside increased the cell viability at 25 μg/ml.

In conclusion, acteoside, PhGs and echinacoside exerted significant neuroprotective effects on PC12 cells subjected to injury with Aβ₁₋₄₂ or H₂O₂.

Discussion

Oxidative stress is the major mechanism underlying Aβ-mediated neurotoxicity in AD (11-13). Therefore, targeting oxidative stress may represent an approach for the treatment of AD. In the present study, an *in vitro* model of AD comprising Aβ₁₋₄₂- and H₂O₂-induced PC12 cell injury was successfully established. Results of the MTT, LDH and MDA assays showed that PhGs increased the cell viability, and decreased LDH and MDA release by PC12 cells subjected to injury. It can be concluded that PhGs have significant neuroprotective effects on PC12 cells.

In order to reduce the effects of PhGs themselves on PC12 cell growth and prevent abnormal proliferation, the safe dose of PhGs was determined in a screening assay. The results showed that PhGs at 75, 100, 125, 150, 175 and 200 μg/ml had a significant inhibitory effect on PC12 cells (P<0.05, P<0.01), while cell viability remained >80% at concentrations of 5, 25 and 50 μg/ml. Thus, PhGs at the concentration of 5, 25 and 50 μg/ml were safe for PC12 cells.

The injury by Aβ₁₋₄₂ was affected by certain factors, including the solvent, incubation time and product quality. In the present study, Aβ₁₋₄₂ peptide was dissolved in water (100 μg/ml) and incubated at 37°C for 4 days in a CO₂ incubator prior to use. PC12 cells were treated with Aβ₁₋₄₂ at

concentrations of 0.5, 1, 1.5 and 2 μM. The results showed that the cell viability was decreased with the increase of Aβ₁₋₄₂, and the viability was <50% in the 1, 1.5 and 2 μM Aβ₁₋₄₂ injury groups. Thus, treatment of PC12 cells with 0.5 μM Aβ₁₋₄₂ for 48 h was determined to be the optimal condition for establishing the AD model. Aβ₂₅₋₃₅ has been commonly used to establish AD models due to low cost and simple operation (27-29). The neurotoxicity of Aβ₁₋₄₂ is significantly higher than that of Aβ₂₅₋₃₅, and Aβ₁₋₄₂ is therefore the optimal Aβ fragment for establishing an AD model (1,8-10).

H₂O₂ is an oxidizer and excessive H₂O₂ may cause oxidative damage and induce cell apoptosis (30). In the present study, PC12 cells were treated with 25-500 μM H₂O₂ dissolved in DMEM with or without PBS. The results showed that H₂O₂ dissolved in DMEM without PBS caused abnormal proliferation of PC12 cells. Thus, treatment of PC12 cells with 25 μM H₂O₂ dissolved in DMEM with PBS was the optimal condition for establishing the AD model. Aβ₁₋₄₂-induced injury was greater than H₂O₂-induced injury due to poor stability of H₂O₂ and solvent effects.

When the cell is damaged, LDH leakage into the culture medium is significantly increased. ROS is known to cause the production of MDA. The content of MDA and LDH therefore reflect the amount of oxidative damage. In the present study, damage-induced LDH and MDA activity was decreased with increasing doses of PhGs. These results indicated that PhGs have a significant neuroprotective effect on PC12 cells. The MTT assay showed that PhGs exhibited a dose-dependent neuroprotective effect on PC12 cells.

In conclusion, an *in vitro* model of AD comprising Aβ₁₋₄₂- and H₂O₂-induced PC12 cell injury was successfully established. Treatment with PhGs increased the cell viability, and decreased LDH and MDA release by PC12 cells treated with Aβ₁₋₄₂ or H₂O₂. PhGs had a significant neuroprotective effect on Aβ₁₋₄₂- or H₂O₂-induced cell injury.

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