Di-Huang-Yi-Zhi herbal formula attenuates amyloid-β-induced neurotoxicity in PC12 cells

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Received January 17, 2016; Accepted January 13, 2017

DOI: 10.3892/etm.2017.4368

Abstract. Traditional Chinese medicine can be used for Alzheimer's disease management, such as the modern herbal formula Di-Huang-Yi-Zhi (DHYZ). In the present study, neuronal differentiated PC12 cells were used as a model to evaluate the effects of DHYZ against amyloid-β peptide 25-35 ($A\beta_{25-35}$) induced neurotoxicity, particularly regarding cell proliferation, apoptosis and related events. Following treatment with DHYZ, cell viability, cell membrane damage, apoptosis, mitochondrial membrane potential, cytochrome c release, caspase-3 activity and levels of reactive oxygen species in PC12 cells were detected. The results demonstrated that pretreatment with DHYZ significantly protected PC12 cells from $A\beta_{25-35}$-induced proliferation inhibition, lactate dehydrogenase release and apoptosis, as well as upregulating mitochondrial membrane potential and downregulating cytochrome *c* release and caspase-3 activation. DHYZ also inhibited the $A\beta_{25-35}$-induced reactive oxygen species generation in PC12 cells. These observations suggest that DHYZ protected PC12 cells from the Aβ-induced neurotoxicity.

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Key words: amyloid-β, PC12 cells, Di-Huang-Yi-Zhi, apoptosis, mitochondria, reactive oxygen species

Introduction

Alzheimer's disease (AD), the most frequent cause of dementia, is an age-associated progressive neurodegenerative disorder characterized by memory loss and cognitive decline. Amyloid-β (Aβ)-induced neurotoxicity and oxidative stress contribute to AD pathogenesis (1,2). Therefore, Aβ-induced neuronal cell damage is widely used as a model to investigate neuroprotective agents. Currently approved drugs, including donepezil, galantamine, rivastigmine and memantine, only demonstrate marginal therapeutic benefits for AD (3). Thus, there is an urgent requirement to develop novel approaches for AD treatment.

Traditional Chinese medicine (TCM) formulas can be a valuable medical and pharmaceutical resource for AD management. TCM herbal treatments, including Tiao-Xin-Fang, Bu-Shen-Fang and Yi-zhi-Fang-Dai granules, have demonstrated efficacy in improving cognitive and brain function, and in ameliorating clinical symptoms in AD patients (4,5). Combined Chinese and Western medicine has shown better efficacy compared with modern drug therapy alone in AD treatment (6). Based on the TCM principles and studies in Chinese herbal pharmacology, previous studies by our group established a Di-Huang-Yi-Zhi (DHYZ) formula, mainly comprised of the herbs Shu-Di, Dan-Shen, Yi-Zhi-Ren, Fu-Shen and Shi-Chang-Pu (Chinese patent no. ZL2008102047153.3), for the treatment of AD (7,8).

DHYZ herbal extracts have been demonstrated to be effective in improving the learning and memory abilities in rats with dementia induced by Aβ that is accompanied by decreased choline acetyltransferase activities and increased acetylcholinesterase activities (7). DHYZ also improves the daily life and cognitive abilities, and relieves clinical symptoms, while it has shown enhanced efficacy when combined with donepezil in AD patients (8). Since Aβ mediated neurotoxicity is closely related to the pathogenesis of AD, the current study used neuronal differentiated PC12 cells treated with Aβ as a model of Aβ neurotoxicity to evaluate the potential neuroprotective effects of DHYZ herbs.

Materials and methods

Chemicals and reagents. Aβ protein fragment 25-35 ($A\beta_{25-35}$; cat. no. A4559-1MG) was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Recombinant rat nerve growth factor (NGF) and Rat/Mouse Cytochrome *c* (Cyt-C) ELISA kits (cat. nos. 7815-NG and MCTC0, respectively) were obtained from R&D Systems (Minneapolis, MN, USA). A Cell Counting kit-8 (CCK-8; cat. no. CK04) was purchased from Dojindo (Kumamoto, Japan) and a Cell Death Detection ELISA^{Plus} kit (cat. no. 11774425001) was purchased from Roche Applied Sciences (Basel, Switzerland). A lactate dehydrogenase (LDH) Cytotoxicity Assay kit was obtained from Cayman Chemical (cat. no. 601170; Ann Arbor, MI, USA). A BCA Protein assay kit (cat. no. P0010S), mitochondrial membrane potential assay kit (cat. no. C2006), caspase-3 activity assay kit (cat. no. C1116), cytoplasmic protein extraction kit (cat. no. P0028) and 2',7'-dichlorofluorescin diacetate (DCFH-DA; cat. no. S0033) were provided by Beyotime Institute of Biotechnology (Jiangsu, China).

Preparation of herbal extract. The main herbs in the DHYZ formula are processed root of *Rehmannia glutinosa* (Gdertn) Libosch. (Shu-Di), the root of *Salvia miltiorrhiza* Bge (Dan-Shen), the fruits of *Alpinia oxyphylla* Miq. (Yi-Zhi-Ren), *Poria* with hostwood (Fu-Shen) and the root of *Acorus tatarinowii* Schott (Shi-Chang-Pu) (Chinese patent no. ZL2008102047153.3). Extraction and quality control of DHYZ was performed as previously described (9-11). Briefly, the herbs were identified by thin layer chromatography, and salvianolic acid B was used as an indicator for quality control. Herbs were extracted first with a 10-fold volume of boiling distilled water for 1 h, then with a 6-fold volume of boiling distilled water for 1 h. Two aqueous extracts of the herbs were combined and precipitated with 50% ethanol for 24 h, centrifuged at 5,000 x *g* for 10 min at 4˚C, then evaporated in vacuum conditions for 12 h at 60˚C. Subsequently, the ethanol extracts were dissolved in RPMI 1640 medium (Thermo Fisher Scientific, Inc.), sequentially passed through 0.45 and 0.22 μm filters for sterilization, and stored at 4˚C until further use.

Aβ preparation. $A\beta_{25-35}$ aggregation was prepared as described previously (12). Briefly, lyophilized $A\beta_{25-35}$ was dissolved in RPMI 1640 medium and incubated at 37˚C with constant oscillation for 3 days in order to induce aggregation. The aggregated $A\beta_{25-35}$ was then diluted to 100 μg/ml (100 μM) and stored at -20˚C until required.

Cell culture. PC12 cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). PC12 cells were grown in RPMI 1640 medium with 10% horse serum (cat. no. 16050122), 5% fetal bovine serum (cat. no. 10099-141) and 1% Pen-Strep (cat. no. 15140122; all from Thermo Fisher Scientific, Inc., Waltham, MA, USA), and maintained at 37˚C in a humidified incubator in a 5% CO_2 atmosphere for 2-3 d. PC12 cells were treated with NGF (50 ng/ml) for 2 days at 37˚C to induce neuronal differentiation, and subsequently examined by a microscope (13,14). Differentiated PC12 cells were used in all experiments.

Cell viability assay. Differentiated PC12 cells were seeded into 96-well plate ($8x10^3$ cells/well) and grown in complete RPMI 1640 medium for 24 h at 37˚C. Cells were then pretreated with various doses of DHYZ (10, 20 and 40 μg/ml) for 1 h at 37˚C, followed by 20 μM aggregated $A\beta_{25-35}$ treatment for 24 h at 37˚C. Cells treated with the same volume of RPMI 1640 medium alone were used as a control. At the end of the treatment, cell viability was evaluated by a CCK-8 assay according to the manufacturer's instructions. Cell viability rate was calculated on the basis of the optical density (OD), as follows: Cell viability (%) = (Experimental OD value/control OD value) x100%.

LDH release assay. Differentiated PC12 cells were seeded and treated as described for the cell viability assay. At the end of the treatment, $A\beta_{25-35}$-induced cytotoxicity was evaluated by an LDH release assay, according to the manufacturer's instructions. The results are expressed as the fold change compared with the control group.

Quantification of cell apoptosis. Differentiated PC12 cells were seeded and treated as described for the cell viability assay. At the end of the treatment, PC12 cells were collected for quantification of DNA fragmentation using the Cell Death Detection ELISA^{Plus} kit, according to the manufacturer's protocol. Results are expressed as the fold change compared with the non-treated control.

Measurement of mitochondrial membrane potential (MMP). Differentiated PC12 cells were seeded into 24-well plate ($4x10^4$ cells/well) and grown in complete RPMI 1640 medium for 24 h at 37˚C. Cells were then pre-incubated with or without DHYZ (10, 20 and 40 μg/ml) for 1 h at 37˚C, followed by exposure to 20 μM aggregated $A\beta_{25-35}$ for 24 h at 37˚C. Cells treated with with the same volume of RPMI 1640 medium alone were used as control. MMP changes were determined using the MMP assay kit, according to the manufacturer's protocol. The presence of JC-1 fluorescence was observed under a fluorescence microscope.

Cyt-C release assay. The release of Cyt-C from mitochondria into the cytosol was assessed by ELISA. Differentiated PC12 cells were seeded into 6-well plate ($2x10^5$ cells/well) and grown in complete RPMI 1640 medium for 24 h at 37˚C. Cells were then pretreated with or without DHYZ (10, 20 and 40 μg/ml) for 1 h at 37˚C, after which the cells were exposed to 20 μM aggregated $A\beta_{25-35}$ for 24 h at 37˚C. Cells treated with the same volume of RPMI 1640 medium alone were used as a control. After treatment, PC12 cells were collected and subjected to cytoplasmic protein extraction according to the manufacturer's protocol of the corresponding kit. Cytosolic Cyt-C was measured using the Rat/Mouse Cyt-C ELISA kit, according to the manufacturer's manual. The results are expressed as the fold change in comparison with the non-treated control.

Caspase-3 activity assay. Differentiated PC12 cells were seeded and treated as described for the Cyt-C release assay. Following treatment, PC12 cells were collected and subjected to caspase-3 activity detection by the cleavage of the specific chromogenic

substrate according to the manufacturer's instructions of the kit. The results are expressed as the fold change in caspase-3 activity in comparison with the non-treated control.

Measurement of reactive oxygen species (ROS) generation. Intracellular ROS production was detected by DCFH-DA staining. DCFH-DA is cleaved intracellularly by nonspecific esterases to form DCFH, which is further oxidized by ROS to form the fluorescent compound DCF. Differentiated PC12 cells were pre-incubated with or without DHYZ and exposed to aggregated $A\beta_{25\text{-}35}$, as described for the aforementioned MPP assay. Cells were then stained with DCFH-DA at 37˚C for 20 min in the dark. DCF fluorescence was quantified with a fluorescence microplate reader at an excitation wavelength of 488 nm and emission wavelength of 525 nm. The results are expressed as the fold change compared with the non-treated control.

Statistical analysis. Results are expressed as the mean ± standard deviation of at least two independent experiments, each conducted in triplicate. Differences between the control and treatment groups were analyzed by one-way analysis of variance. Differences were considered to be statistically significant at $P<0.05$.

Results

Effect of DHYZ on the cell viability in Aβ-treated PC12 cells. $A\beta_{25\text{-}35}$-treated neuronal differentiated PC12 cells were used as a model to evaluate neuroprotective effects of DHYZ. The effects of DHYZ on cell viability in these cells were observed by CCK-8 assay. As shown in Fig. 1, $A\beta_{25\text{-}35}$ significantly inhibited PC12 cell growth compared with the control ($P<0.01$). By contrast, DHYZ treatment significantly increased the cell viability of $A\beta_{25\text{-}35}$-treated PC12 cells in a dose-dependent manner relative to the $A\beta_{25\text{-}35}$ group ($P<0.01$), and the effects of 40 μg/ml DHYZ were significantly greater than 10 and 20 μg/ml DHYZ ($P<0.05$).

Effect of DHYZ on LDH release in Aβ-treated PC12 cells. LDH, a soluble cytosolic enzyme, is released from cells following the loss of cell membrane integrity due to cell death. LDH release was used as an indicator for $A\beta_{25\text{-}35}$-induced cytotoxicity. As shown in Fig. 2, $A\beta_{25\text{-}35}$ treatment significantly increased LDH leakage in PC12 cells compared with the non-treated control group ($P<0.01$). However, pretreatment with 10-40 μg/ml of DHYZ significantly decreased the $A\beta_{25\text{-}35}$-elicited LDH release in PC12 cells in a dose-dependent manner ($P<0.05$), and the effect of 40 μg/ml DHYZ in reducing LDH release was significantly greater than that observed for 10 and 20 μg/ml DHYZ ($P<0.05$).

Effect of DHYZ on Aβ-induced apoptosis in PC12 cells. The effect of DHYZ on cell death in $A\beta_{25\text{-}35}$-treated PC12 cells was measured by ELISA, which specifically detects cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes), which indicate apoptosis. The results indicated that $A\beta_{25\text{-}35}$ significantly induced apoptosis in PC12 cells compared with the control group ($P<0.01$). However, pretreatment with 10-40 μg/ml DHYZ significantly protected PC12 cells from $A\beta_{25\text{-}35}$-induced apoptosis in a dose-dependent manner ($P<0.05$), and the protective effects of 40 μg/ml DHYZ were

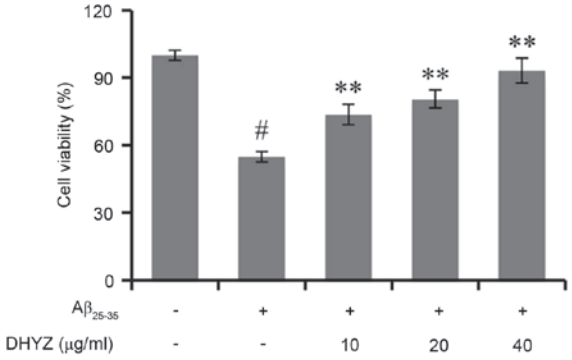

Figure 1. Effect of DHYZ on the cell viability in Aβ-treated PC12 cells. Differentiated PC12 cells were pretreated with different concentrations of DHYZ for 1 h, followed by $A\beta_{25\text{-}35}$ treatment for 24 h. Cell viability was evaluated by CCK-8 assay. Data shown are representative of three independent experiments. #P<0.01, vs. non-treated group; **P<0.01 vs. $A\beta_{25\text{-}35}$ group. DHYZ, Di-Huang-Yi-Zhi; Aβ, amyloid-β; CCK-8, cell counting kit-8.

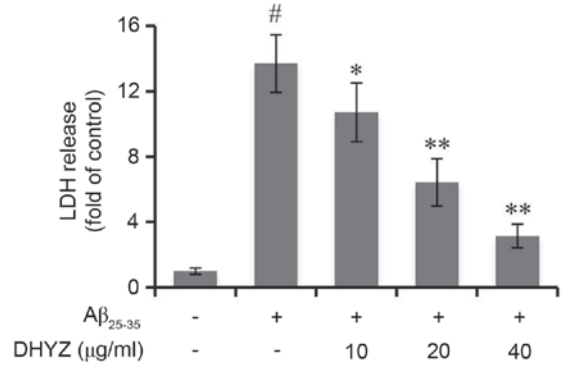

Figure 2. Effect of DHYZ on the LDH release in Aβ-treated PC12 cells. Differentiated PC12 cells were pretreated with different concentrations of DHYZ for 1 h, followed by $A\beta_{25\text{-}35}$ treatment for 24 h. LDH release was determined by an LDH Cytotoxicity Assay kit, and is expressed as the fold of the control. Data are from three independent experiments. #P<0.01 vs. non-treated group; *P<0.05 and **P<0.01 vs. $A\beta_{25\text{-}35}$ group. DHYZ, Di-Huang-Yi-Zhi; Aβ, amyloid-β; LDH, lactate dehydrogenase.

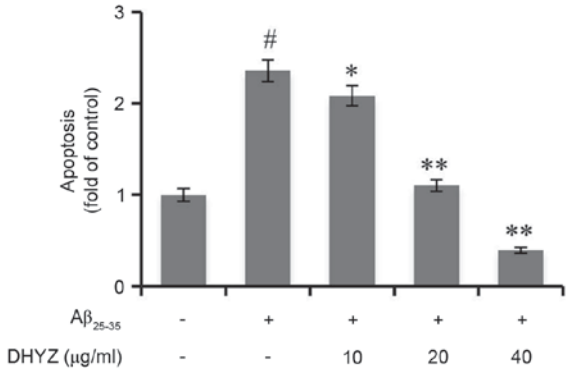

Figure 3. Effect of DHYZ on Aβ-induced apoptosis in PC12 cells. Differentiated PC12 cells were pretreated with different concentrations of DHYZ for 1 h, followed by $A\beta_{25\text{-}35}$ treatment for 24 h. Cell death was detected by ELISA. Results are expressed the as fold of the non-treated control, and were obtained from three separate experiments. #P<0.01 vs. non-treated group; *P<0.05 and **P<0.01, vs. $A\beta_{25\text{-}35}$ group. DHYZ, Di-Huang-Yi-Zhi; Aβ, amyloid-β.

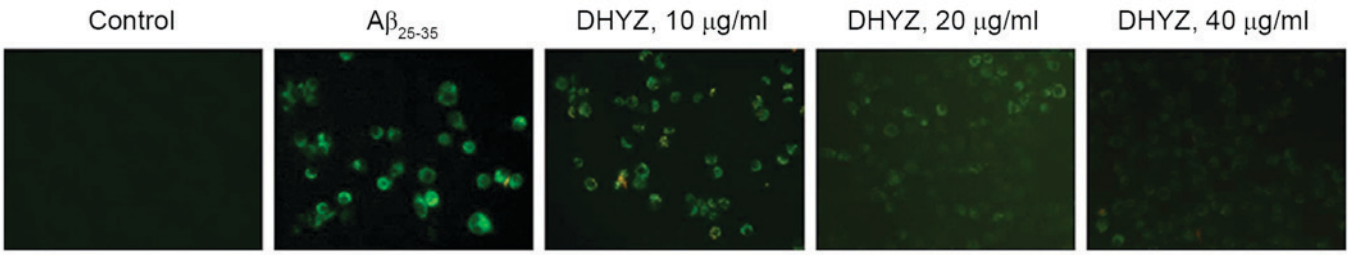

Figure 4. Effect of DHYZ on Aβ-induced MMP change in PC12 cells. Differentiated PC12 cells were pretreated with different concentrations of DHYZ for 1 h, followed by $A\beta_{25-35}$ treatment for 24 h. MMP was detected by JC-1 staining, and observed under a fluorescence microscope (magnification, x200). DHYZ, Di-Huang-Yi-Zhi; Aβ, amyloid-β; MMP, mitochondrial membrane potential.

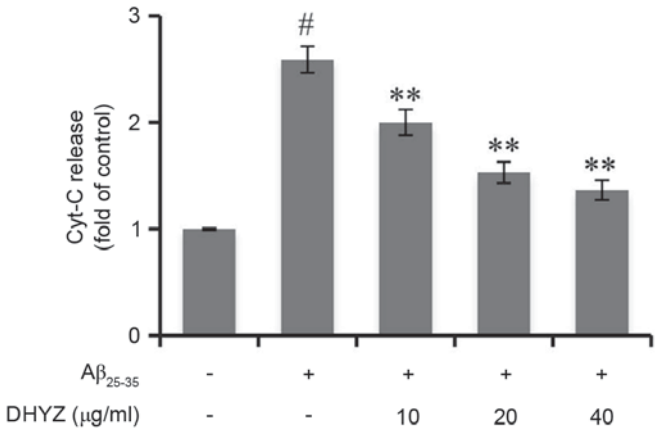

Figure 5. Effect of DHYZ on Aβ-elicited Cyt-C release in PC12 cells. Differentiated PC12 cells were pre-incubated with different concentrations of DHYZ for 1 h, followed by $A\beta_{25-35}$ treatment for 24 h. Cyt-C concentration in the cytosol was determined by ELISA. Data are presented from three separate experiments. #P<0.01 vs. non-treated group; **P<0.01 vs. $A\beta_{25-35}$ group. DHYZ, Di-Huang-Yi-Zhi; Aβ, amyloid-β; Cyt-C, cytochrome *c*.

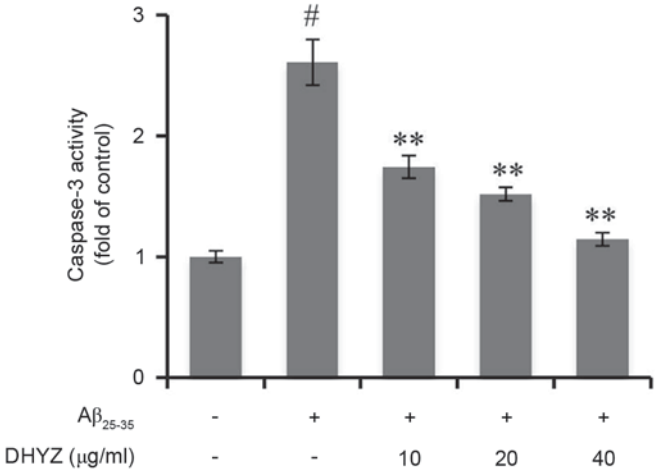

Figure 6. Effect of DHYZ on caspase-3 activity in $A\beta_{25-35}$-treated PC12 cells. Differentiated PC12 cells were pretreated with different concentrations of DHYZ for 1 h, followed by $A\beta_{25-35}$ treatment for 24 h. Caspase-3 activity was detected using a kit, and is expressed as the fold of the control. Data shown are representative of three independent experiments. #P<0.01 vs. non-treated group; **P<0.01 vs. $A\beta_{25-35}$ group. DHYZ, Di-Huang-Yi-Zhi; Aβ, amyloid-β.

significantly greater than those observed for 10 and 20 μg/ml DHYZ (P<0.01; Fig. 3).

Effects of DHYZ on Aβ-induced MMP change in PC12 cells. JC-1 was used to detect MMP as a parameter of mitochondrial function. JC-1 is a lipophilic cationic dye that remains in monomeric form and yields green fluorescence in apoptotic cells with low MMP. $A\beta_{25-35}$ treatment reduced the MMP in PC12 cells, as observed by the increased green fluorescence. By contrast, pre-incubation with 10-40 μg/ml of DHYZ countered the lowering MMP induced by $A\beta_{25-35}$ as indicated by reduced fluorescence (Fig. 4).

Effects of DHYZ on Aβ-induced Cyt-C release in PC12 cells. Mitochondrial dysfunction and subsequent Cyt-C release are central events in apoptosis (15). In the present study, the Cyt-C concentration in the cytosol was measured by ELISA. PC12 cells treated with $A\beta_{25-35}$ showed an increase in cytosolic Cyt-C levels compared with the control levels (P<0.01). Pretreatment with 10-40 μg/ml DHYZ significantly attenuated $A\beta_{25-35}$-induced Cyt-C release in a dose-dependent manner (P<0.01), and the effect of 40 μg/ml DHYZ in reducing Cyt-C release was significantly greater than that observed for 10 μg/ml DHYZ (P<0.01; Fig. 5).

Effect of DHYZ on caspase-3 activity in Aβ-treated PC12 cells. Cell apoptosis is executed by a caspase cascade. Caspase-3 is the final executor of apoptosis, and its activation is a hallmark of apoptosis (16). As shown in Fig. 6, $A\beta_{25-35}$ treatment activated caspase-3 in PC12 cells when compared with the control group (P<0.01). Pretreatment with 10-40 μg/ml DHYZ significantly inhibited the $A\beta_{25-35}$-induced caspase-3 activation in PC12 cells in a dose-dependent manner (P<0.01), and the effect of 40 μg/ml DHYZ in supressing caspase-3 activation was significantly greater than that observed for 10 and 20 μg/ml DHYZ (P<0.01).

Effect of DHYZ on Aβ-induced ROS generation in PC12 cells. Aβ-mediated ROS generation contributes to AD pathogenesis (17,18). As shown in Fig. 7, exposure of PC12 cells to $A\beta_{25-35}$ for 24 h significantly increased ROS generation as compared with the control cells (P<0.01). Pretreatment with 20

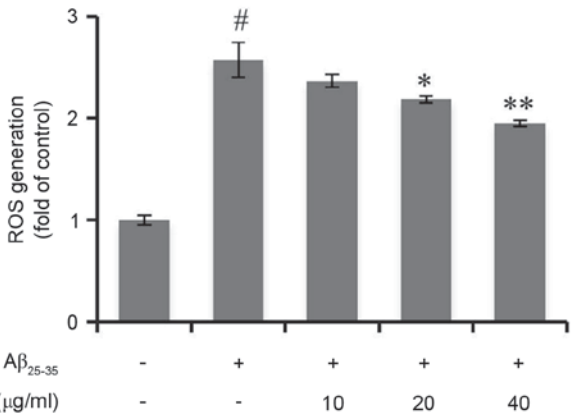

Figure 7. Effect of DHYZ on $A\beta_{25-35}$-induced ROS generation in PC12 cells. Differentiated PC12 cells were pretreated with different concentrations of DHYZ for 1 h, followed by Aβ25-35 treatment for 24 h. The production of intracellular ROS was detected by DCFH-DA staining and quantified with a fluorescence microplate reader (excitation wavelength, 488 nm; emission wavelength, 525 nm). ROS production was expressed as the fold of the control group. Data shown are representative of three independent experiments. $^{\#}P<0.01$ vs. non-treated group; $^{*}P<0.05$ and $^{**}P<0.01$, vs. $A\beta_{25-35}$ group. DHYZ, Di-Huang-Yi-Zhi; Aβ, amyloid-β; ROS, reactive oxygen species; DCFH-DA, 2',7'-dichlorofluorescin diacetate.

and 40 μg/ml of DHYZ significantly reduced the ROS production ($P<0.05$), and the effect of 40 μg/ml DHYZ in reducing ROS was significantly greater than that observed for 10 and 20 μg/ml DHYZ ($P<0.05$). Collectively, these observations suggest that DHYZ exerts neuroprotective effects against Aβ-induced neurotoxicity, which may contribute to the therapeutic effects of DHYZ in the treatment of AD.

Discussion

According to TCM theory and clinical observations, the pathogenesis of AD is closely associated with kidney deficiency resulting in brain malnutrition, blood stasis and phlegm retention (7,19). In the DHYZ formula, Shu-Di is used as a Monarch (Jun) herb for nourishing the kidney. The Dan-Shen and Shi-Chang-Pu in the DHYZ formula are used as Minister (Chen) for dissolving stasis and phlegm, and calming the mental state. Fu-Shen is used as an Assistant (Zuo) herb to calm the nerves, while Yi-Zhi-Ren is used as a Guide (Shi) herb to warm the kidney.

Certain herbs in the DHYZ formula have been demonstrated to be beneficial in AD. Shu-Di is one of the most frequently used herbs for AD treatment, and has been shown to improve learning and memorizing ability in aluminum chloride-induced dementia mice (20). Shu-Di can also inhibit p53, p21 and p16 expression, protect brain tissue from oxidation and delay brain senescence in D-galactose-induced senile rats (21,22). Catalpol, a compound in Shu-Di, has been demonstrated to attenuate the $A\beta_{1-42}$-mediated neurotoxicity, improve the endocrine function and alleviate structural damage of the hypothalamus in $A\beta_{25-35}$-induced AD rats (23,24).

Dan-Shen inhibits acetylcholinesterase and protects PC12 cells from Aβ-induced cytotoxicity (25). In addition, Shi-Chang-Pu protects PC12 cells from $A\beta_{25-35}$-induced cell damage, mitochondrial dysfunction, ROS generation and cell death (26). Yi-Zhi-Ren has been demonstrated to have protective effects on learning and memory impairments, neuronal damage and apoptosis induced by $A\beta_{1-42}$ (27). 5-Hydroxymethylfurfural, a compound isolated from Yi-Zhi-Ren, ameliorates learning and memory impairment, inhibits β-secretase activity, increases antioxidative enzyme activities and mitigates neuronal damage in $A\beta_{1-42}$-induced AD mice (28).

Extracellular amyloid plaques have been identified as one of the pathological characteristics in the AD brain, and amyloid-mediated neuron damage is a major cause of AD (29). In the present study, $A\beta_{25-35}$ was found to significantly reduce cell viability and increase LDH release due to membrane damage, which confirmed the neurotoxicity of $A\beta_{25-35}$ on neuronal differentiated PC12 cells. DHYZ pretreatment significantly reversed these changes induced by $A\beta_{25-35}$, suggesting that DHYZ has neuroprotective effects against $A\beta_{25-35}$-mediated toxicity and may be beneficial for AD treatment.

Aβ induces mitochondrial dysfunction leading to the release of Cyt-C, which forms an apoptosome and activates the initiating protease caspase-9, which in turn activates the executioner caspases-3, and ultimately results in apoptosis (30,31). In the present study, $A\beta_{25-35}$ treatment downregulated the MMP, increased the mitochondrial Cyt-C release and activated caspase-3 accompanied by apoptosis induction in PC12 cells. Pretreatment with DHYZ inhibited these changes, suggesting that DHYZ protected PC12 cells from $A\beta_{25-35}$-induced apoptosis.

The Aβ peptide may also increase ROS generation (32). A high level of ROS promotes cell death via the mitochondrial pathway and/or death receptor pathway (33). Components in the DHYZ formula, such as catalpolcatalpol, tanshinone IIA and Shi-Chang-Pu extract, may inhibit Aβ-induced ROS generation (26,34,35). In the present study, DHYZ pretreatment was observed to attenuate the Aβ-induced ROS production in PC12 cells, suggesting that ROS inhibition may contribute to DHYZ-mediated neuroprotective effects.

In conclusion, the present study demonstrated that DHYZ exerted neuroprotective effects against $A\beta_{25-35}$-mediated neurotoxicity by alleviating $A\beta_{25-35}$-induced proliferation inhibition, apoptosis and ROS generation. The present study provides new insight into the application of Chinese herbs for AD treatment that are worthy of further investigation.

Acknowledgements

The present study was supported by grants from the National Natural Science Foundation of China (no. 81470195), the Research Project from Shanghai Municipal Health and Family Planning Commission (no. 2014XZ005A), the Three-year Action Program of Shanghai Municipality for Traditional Chinese Medicine (no. ZY3-JSFC-2-2003), and the Opening Research Fund of Shanghai Key Laboratory of Tissue Engineering (no. TE1401).

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