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Research paper

Protective effects of a Chotosan Fraction and its active components on β-amyloid-induced neurotoxicity

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HIGHLIGHTS

- A Chotosan fraction, CTS-E, had significant protective effects on Aβ-induced neurotoxicity.
- Chemical profile of CTS-E was determined by LC–MS.
- Phenolic acids from *Chrysanthemi Flos* and flavonoids from *Citri Reticulatae Pericarpium* might be the effective constituents in CTS-E.

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ABSTRACT

Chotosan (CTS) is a traditional Kampo prescription used to treat chronic headache and hypertension. Recent clinical studies demonstrated that CTS has ameliorative effects on dementia. This study aims to identify the anti-Alzheimer components in CTS. β-amyloid (Aβ) is considered to play a central role in the pathophysiology of Alzheimer's disease. CTS-E, a fraction of CTS, showed significant protective effects on Aβ-induced neurotoxicity. High-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry was used for the qualitative analysis of it. Among the identified constituents, neuroprotective effects against $A\beta_{25-35}$-induced neurotoxicity of 10 major compounds were tested by MTT assay. Their inhibitory action on $A\beta_{1-42}$ self-induced aggregation was measured by Thioflavin T-binding assay. The results showed that caffeic acid, chlorogenic acid, 1,5-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid had significant neuroprotective effects on $A\beta_{25-35}$-induced neurotoxicity. Besides these phenolic acids, nobiletin and hesperidin could also inhibit $A\beta_{1-42}$ self-induced aggregation. In conclusion, the neuroprotective fraction, CTS-E, could protect PC12 cells from Aβ-induced neurotoxicity. Anti-oxidative effects may at least partly mediate the neuroprotective effects of it. Phenolic acids from *Chrysanthemi Flos* and flavonoids from *Citri Reticulatae Pericarpium* might be the effective constituents in CTS-E.

1. Introduction

Alzheimer's disease (AD), a kind of dementia, is a problematic disease that has shown a noticeable increase in the number of patients all over the world [1]. One of the characteristics of AD patients is the amyloid plaques in brain, which are the dense deposits of β-amyloid (Aβ). Accumulation of aggregated amyloid fibrils, which is believed to be neurotoxic, is considered to play a central role in the pathophysiology of AD [2–4].

Chotosan (CTS) is a traditional Kampo prescription, which has been widely used to treat chronic headache and hypertension in China, Japan, and Korea. Modern clinical and pharmacological studies demonstrated the value of CTS for the treatment of dementia

Abbreviations: CTS, chotosan; CTS-E, chotosan ethyl acetate fraction; AD, Alzheimer's disease; Aβ, β-amyloid; QTOFMS, quadrupole time-of-flight mass spectrometry; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; ThT, thioflavon T; ROS, reactive oxygen species; DAD, diode array detector; ESI, electrospray ionization interface; TIC, total ion chromatograms.

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[1]. In double-blind, randomized, placebo-controlled clinical studies, CTS was effective in treating dementia of both vascular and Alzheimer type [5,6]. In animal studies, CTS showed ameliorative effects on dementia induced by diabetes, ischemia or senescence [7–11]. However, the mechanism of its ameliorative effects on AD remains unknown.

CTS is composed of *Uncariae cum Uncis Ramulus* and other ten medicinal herbs. Although a number of studies have reported the chemical components of each single herb, only few literatures involved the whole prescription [12,13]. Meanwhile, it is uncertain whether the identified compounds are effective in the treatment of AD. On the other hand, the effects of *Uncariae cum Uncis Ramulus* and its active ingredients on AD have been confirmed by lots of studies [14–16], while the effects of other herbs in CTS have not been revealed. The long Chinese medicine usage history has demonstrated that herb compatibility acts better than a single herb [17]. The function of other ten herbs should not be neglected.

In the present study, PC12, a clonal cell line derived from a pheochromocytoma of the rat adrenal medulla, which expresses neuronal properties, was used as the model system. The complicated formula was separated into several fractions according to their differences in polarity by a solvent extraction system, aiming at rapid exploration of the active anti-Alzheimer components. We found that the fraction, CTS-E, showed better neuroprotective effects than other fractions. To clarify the chemical profile of this fraction, high-performance liquid chromatography with quadrupole time-of-flight mass spectrometry (HPLC-QTOFMS) method was established for its qualitative analysis. Among the identified constituents, neuroprotective effects against $A\beta_{25-35}$ induced cytotoxicity and inhibitory action on self-induced $A\beta_{1-42}$ aggregation of 10 major compounds were tested.

2. Materials and methods

2.1. Herb materials and extraction

CTS is composed of 11 crude drugs: 3.0 parts *Uncariae cum Uncis Ramulus*, 3.0 parts *Citri Reticulatae Pericarpium*, 3.0 parts *Pinelliae Tuber*, 3.0 parts *Ophiopogonis Radix*, 3.0 parts *Poria*, 2.0 parts *Ginseng Radix*, 2.0 parts *Saposhnikoviae Radix*, 2.0 parts *Chrysanthemi Flos*, 1.0 part *Glycyrrhizae Radix*, 1.0 part *Zingiberis Rhizoma*, and 5.0 parts *Gypsum fibrosum* ($CaSO_4{\cdot}2H_2O$). They were supplied by Jiangxi Qingfeng Pharmaceutical Co., Ltd. (Ganzhou, Jiangxi, China). These herbal materials were identified by Professor Feng, the botanist in Department of Phytochemistry of our university.

Totally about 9.0 kg of the 11 herbs were mixed in the ratio above. The mixture was refluxed successively with water (100 °C, 2 h), 95% ethanol (80 °C, 2 h), and 50% ethanol (80 °C, 2 h). The extracts were separated from insoluble waste, concentrated and extracted with ethyl acetate three times. The ethyl acetate layers were combined and dried by vacuum distillation to get the fraction of CTS-E (yield: 116 g). The aqueous layer was eluted through D-101 macroporous adsorption resin. We collected the fractions from H_2O-ethanol (100: 0, 80: 20, 60: 40, 30: 70, 5: 95) and removed the solvent to get the dried extracts of CTS-0, CTS-20, CTS-40, CTS-70, CTS-95 fractions, respectively (yield: 367 g, 189 g, 22 g, 25 g, 6 g).

2.2. Chemicals and reagents

The standard compounds of chlorogenic acid (**3**), caffeic acid (**5**), cimifugin (**11**), narirutin (**13**), 1,5-dicaffeoylquinic acid (**15**), 3,5-dicaffeoylquinic acid (**16**), hesperidin (**19**), 4,5-dicaffeoylquinic acid (**21**), nobiletin (**41**) and tangeretin (**45**) were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Purity of all the compounds was above 98%.

Reagents used in analytical experiments, HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). HPLC-grade water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA). Other reagents were all of analytical grade and purchased from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China). Reagents used in pharmacological experiments, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (GrandIsland, NY, USA). $A\beta_{25-35}$ and $A\beta_{1-42}$ were obtained from Sigma-Aldrich Co. (Shanghai, China). 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) was provided by TCI Development Co., Ltd. (Shanghai, China). Thioflavon T (ThT), resveratrol and curcumin were bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and any other reagents were supplied by Beyotime Institute of Biotechnology (Shanghai, China).

For qualitative analysis, 30 mg of CTS-E powder was accurately weighed and dissolved in 5 mL of methanol in a 30 °C ultrasonic bath for 30 min. The solution was filtered through a 0.45 μm membrane filter before injection. For pharmacological experiments, fractions of CTS were accurately weighed and dissolved in dimethyl sulfoxide (DMSO) at the concentration of 100 mg/mL respectively. The ten standard compounds were prepared by the same way at the concentration of 50 mM respectively. All solutions were stored at 4 °C and diluted to corresponding concentrations before use.

2.3. Preparation of aggregated $A\beta_{25-35}$

$A\beta_{25-35}$ was dissolved in deionized distilled water (1 mM), aggregated at 37 °C for 4 days and stored at −20 °C until use [18].

2.4. Cell culture and drug treatments

PC12 cells (Institute of Biochemistry and Cell Biology, Shanghai, China) were cultured in DMEM containing penicillin (100 U/mL), streptomycin (100 μg/mL) and 10% (v/v) FBS at 37 °C in a humidified atmosphere of 95% air and 5%CO_2. The cells were seeded onto 96-well culture plate at a density of 1.5×10^4 cells/well and allowed to adhere for 12 h. They were subsequently cultured in free serum medium and incubated with different test substances for 0.5 h. $A\beta_{25-35}$ at a final concentration of 10 μM was then added to the culture and incubated for an additional 24 h.

2.5. MTT assay

Cell viability was tested by MTT assay. After drug treatments, MTT in PBS (pH 7.4) was added to each well at a final concentration of 0.5 mg/mL. The plate was incubated at 37 °C for 2 h, followed by the addition of 100 μL DMSO to dissolve the dark blue formazan crystals. Their absorbance at 570 nm was measured on a 1500 microplate reader (Thermo Fisher Scientific Co.). Cell viability was calculated as the percentage of untreated control.

2.6. Measurement of intracellular reactive oxygen species (ROS) production

Intracellular ROS level was detected using DCFH-DA probe [19]. After drug treatments, cells were washed with PBS. They were incubated with DCFH-DA (at the final concentration of 10 μM) in dark at 37 °C for 30 min. Here, nonfluorescent DCFH-DA was enzymatically converted into fluorescent dichlorofluorescein (DCF) in the presence of ROS. Extracellular DCFH-DA was washed off with PBS. The fluorescence intensity of DCF was measured on the 1500 microplate reader at an excitation wavelength of 485 nm and an