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***Yizhijianao* Granule and a combination of its effective monomers, icariin and *Panax notoginseng saponins*, inhibit early PC12 cell apoptosis induced by beta-amyloid (25–35)[☆]**

Ting Zhang¹, Zhanwei Zhang², Keli Dong¹, Guangcheng Li¹, Hong Zhu¹

¹ Department of Traditional Chinese Medicine, Xiangya Third Hospital, Central South University, Changsha 410013, Hunan Province, China

² Department of Neurosurgery, First Affiliated Hospital of Hunan University of Chinese Medicine, Changsha 410007, Hunan Province, China

Abstract

One of our previous studies showed that *Yizhijianao* Granule, a compound Chinese medicine, effectively improved the clinical symptoms of Alzheimer's disease. In the present study, we established a model of Alzheimer's disease using beta-amyloid (25–35) in PC12 cells, and treated the cells with *Yizhijianao* Granule and its four monomers, i.e., icariin, catechin, *Panax notoginseng saponins*, and eleutheroside E. Flow cytometry showed that *Yizhijianao* Granule-containing serum, icariin, *Panax notoginseng saponins*, and icariin + *Panax notoginseng saponins* were protective against beta-amyloid (25–35)-induced injury in PC12 cells. Icariin in combination with *Panax notoginseng saponins* significantly inhibited early apoptosis of PC12 cells with beta-amyloid (25–35)-induced injury compared to icariin or *Panax notoginseng saponins* alone. The effects of icariin + *Panax notoginseng saponins* were similar to the effects of *Yizhijianao* Granule. The findings indicate that two of the effective monomers of *Yizhijianao* Granule, icariin and *Panax notoginseng saponins*, can synergistically inhibit early apoptosis of PC12 cells induced by beta-amyloid (25–35).

Key Words

Alzheimer's disease; icariin; *Panax notoginseng Saponins*; *Yizhijianao* Granule; Chinese medicine monomer; beta-amyloid protein; PC12 cell; Chinese medicine; neural regeneration

Research Highlights

- (1) We screened for the effective concentration of four monomers of *Yizhijianao* Granule.
- (2) Two of the effective monomers of *Yizhijianao* Granule, icariin and *Panax notoginseng saponins*, can synergistically inhibit early PC12 cell apoptosis induced by beta-amyloid (25–35).

Ting Zhang[☆], Studying for doctorate, Attending physician, Department of Traditional Chinese Medicine, Xiangya Third Hospital, Central South University, Changsha 410013, Hunan Province, China

Corresponding author: Keli Dong, Master, Professor, Department of Traditional Chinese Medicine, Xiangya Third Hospital, Central South University, Changsha 410013, Hunan Province, China
dongkeli666@sina.com

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INTRODUCTION

Alzheimer's disease is a multifactorial, complex disease^[1-4]. Multiple target therapy is a novel idea for the treatment of complex diseases. The mechanisms of action of Chinese medicines involve multiple factors, which is consistent to a certain degree with multiple target therapy, and Chinese medicine has been a focus for drug development

for complex diseases^[5].

Clinical studies showed that *Yizhijianao* Granule, a compound Chinese medicine that can invigorate the kidney and promote blood flow, significantly improved cognition and daily life activities of Alzheimer's disease patients, and significantly increased learning, memory and recall abilities of Alzheimer's disease rats^[6-8]. The mechanism of action of *Yizhijianao* Granule may involve the inhibition of nerve

cell apoptosis, oxidative stress and inflammatory reaction in the brain, and the improvement of cholinergic system function and the regulation of β -amyloid protein^[8-13]. Four representative components of *Yizhijianao* Granule, *Yinyanghuo*, the primary active ingredient, Suoyang and Sanqi, secondary ingredients that help strengthen the action of *Yinyanghuo*, and *Ciwujia* (adjuvant drug), were selected to compare the neuroprotective effects of *Yizhijianao* Granule and its active components. These active components are icariin, catechin, *Panax notoginseng saponins*, and eleutheroside E. Drug-containing serum was used in an *in vitro* culture system designed to resemble the *in vivo* environment of cells, to ensure the consistency of experimental results and better reflect the effects of the drug treatments.

However, in contrast with chemical drugs, the quality control of Chinese medicine compounds is complex. We screened effective monomers or monomer combinations from *Yizhijianao* Granule to substitute for the complete and more difficultly controlled *Yizhijianao* Granule.

The present study established an *in vitro* cell model of Alzheimer's disease induced by beta-amyloid (25–35) in PC12 cells. The influence of icariin, catechin, *Panax notoginseng saponins* and eleutheroside E on nerve cell apoptosis was evaluated and the neuroprotective effects of *Yizhijianao* Granule, icariin, catechin, *Panax notoginseng saponins* and eleutheroside E were compared.

RESULTS

Verification of the Alzheimer's disease cell model

PC12 cells were treated with beta-amyloid (25–35) to create an Alzheimer's disease cell model. Inverted phase contrast microscopy showed that normal PC12 cells aggregated and were tightly connected. However, after beta-amyloid (25–35) treatment, the number of viable PC12 cells decreased, the cellular connection was no longer tight, the cytoplasm was dark, the number of cell fragments increased, adherent cells were not transparent, some cells shrank, and a large quantity of particles were observed in the cytoplasm (Figure 1).

Analysis of the effective concentration of the active monomers of *Yizhijianao* Granule on cultured cells

Four concentrations of each monomer were used: 100, 10, 1, and 0.1 mg/mL (icariin, catechin, and eleutheroside E) or 500, 50, 5, and 0.5 mg/mL (*Panax notoginseng saponins*). Compared with that of the control group (normal culture medium), PC12 cell viability (absorbance value in an MTT assay) was significantly enhanced after treatment with 1 mg/mL icariin or 5 mg/mL *Panax notoginseng saponins* ($P < 0.05$), indicating that icariin and *Panax notoginseng saponins* can improve PC12 cell

viability. However, PC12 cell viability remained unchanged in response to treatment with catechin and eleutheroside E for 24 hours (Table 1). Thus, 1 mg/mL icariin and 5 mg/mL *Panax notoginseng saponins* were used in subsequent experiments.

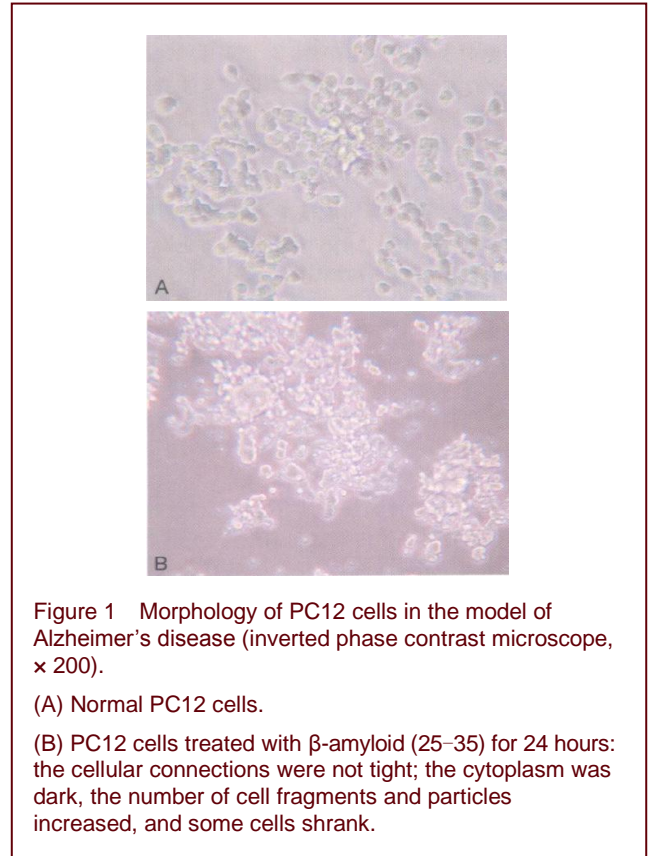


Figure 1 Morphology of PC12 cells in the model of Alzheimer's disease (inverted phase contrast microscope, $\times 200$).

(A) Normal PC12 cells.

(B) PC12 cells treated with β -amyloid (25–35) for 24 hours: the cellular connections were not tight; the cytoplasm was dark, the number of cell fragments and particles increased, and some cells shrank.

Protective effects of *Yizhijianao* Granule monomers on beta-amyloid (25–35)-treated PC12 cells

The Alzheimer's disease cell model was treated with *Yizhijianao* Granule-containing serum, icariin, *Panax notoginseng saponins*, icariin + *Panax notoginseng saponins*, or nerve growth factor (NGF, known to inhibit cell apoptosis, positive control). In addition, normal (normal culture) and model (Alzheimer's disease model) groups were established. An MTT assay showed that PC12 viability (absorbance value) was significantly enhanced in the other groups compared with the model group ($P < 0.01$). PC12 viability (absorbance) was significantly enhanced in the *Yizhijianao* Granule-containing serum and icariin + *Panax notoginseng saponins* groups compared with the icariin alone or *Panax notoginseng saponins* alone groups ($P < 0.05$). There were no significant differences in the viability of the cells in the *Yizhijianao* Granule-containing serum, icariin + *Panax notoginseng saponins*, and NGF groups compared with those in the normal group ($P > 0.05$; Table 2).

Table 1 Effects of the monomers of *Yizhijiannao* Granule on PC12 cell viability

Group	Absorbance value
Control	0.669±0.072
Icariin (mg/mL)	
100	0.676±0.056
10	0.721±0.018
1	0.930±0.051 ^a
0.1	0.623±0.046
Catechin (mg/mL)	
100	0.523±0.010
10	0.672±0.049
1	0.598±0.021
0.1	0.606±0.036
<i>Panax notoginseng saponins</i> (mg/mL)	
500	0.598±0.047
50	0.619±0.016
5	0.927±0.019 ^a
0.5	0.599±0.031
Eleutheroside E (mg/mL)	
100	0.585±0.029
10	0.617±0.013
1	0.598±0.022
0.1	0.563±0.016

^a $P < 0.05$, vs. control group. Data are expressed as mean \pm SD ($n = 5$). One-way analysis of variance and Student-Newman-Keuls tests were used.

Table 2 Effects of *Yizhijiannao* Granule-containing serum and the four monomers on the viability of PC12 cells treated with β -amyloid (25–35)

Group	Absorbance value
Normal	0.436±0.006 ^a
Model	0.281±0.008
<i>Yizhijiannao</i> Granule-containing serum	0.466±0.038 ^{abc}
Icariin	0.381±0.018 ^a
PNS	0.379±0.016 ^a
Icariin+PNS	0.463±0.036 ^{abc}
Nerve growth factor	0.461±0.028 ^a

^a $P < 0.01$, vs. model group; ^b $P < 0.05$, vs. icariin group; ^c $P < 0.05$, vs. PNS group. Data are expressed as mean \pm SD ($n = 5$). One-way analysis of variance and Student-Newman-Keuls tests were used. PNS: *Panax notoginseng saponins*.

Effects of *Yizhijiannao* Granule-containing serum and the four monomers on the apoptosis of PC12 cells treated with beta-amyloid (25–35)

Annexin V-fluorescein isothiocyanate/propidium iodide staining flow cytometry showed that early PC12 cell apoptosis was significantly reduced in the other groups compared with the model group ($P < 0.01$). Early PC12 cell apoptosis was significantly reduced in the *Yizhijiannao* Granule-containing serum and icariin + *Panax notoginseng saponins* groups compared with the icariin or *Panax notoginseng saponins* groups ($P < 0.05$). There were no significant differences in the *Yizhijiannao* Granule-containing serum, icariin + *Panax notoginseng sa-*

ponins, and NGF groups compared with the normal group ($P > 0.05$; Table 3, Figure 2).

Table 3 Effects of *Yizhijiannao* Granule-containing serum and the four monomers on early apoptosis of PC12 cells treated with β -amyloid (25–35)

Group	Percent apoptosis
Normal	4.51±0.36 ^a
Model	28.41±1.65
<i>Yizhijiannao</i> Granule-containing serum	4.82±0.16 ^{abc}
Icariin	8.26±1.30 ^a
PNS	7.79±0.87 ^a
Icariin+PNS	4.90±0.33 ^{abc}
Nerve growth factor	4.79±0.41 ^a

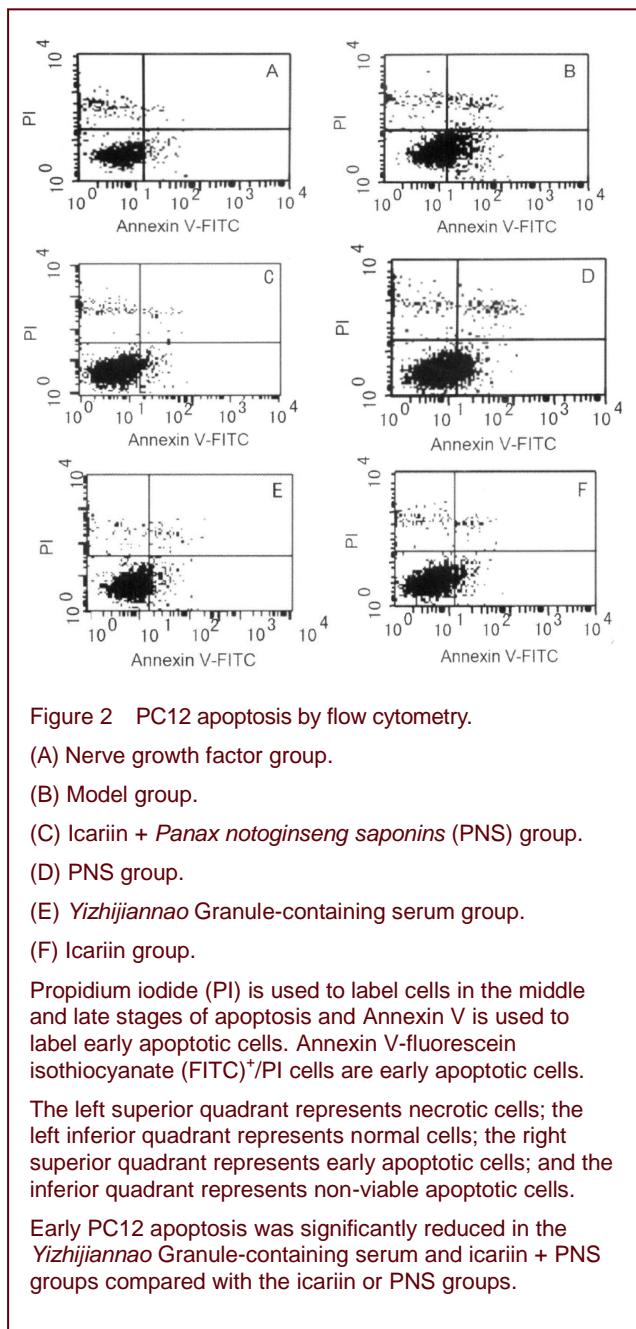
^a $P < 0.01$, vs. model group; ^b $P < 0.05$, vs. icariin group; ^c $P < 0.05$, vs. PNS group. 1×10^5 cells from each sample were detected and the percentage (%) of apoptotic cells was calculated. Data are expressed as mean \pm SD ($n = 5$). One-way analysis of variance and Student-Newman-Keuls tests were used. PNS: *Panax notoginseng saponins*.

DISCUSSION

Results from the present study showed that the icariin + *Panax notoginseng saponins* mixture significantly enhanced PC12 viability compared with other monomers of *Yizhijiannao* Granule, with similar effects to *Yizhijiannao* Granule on protecting PC12 cells from beta-amyloid (25–35) injury. It is likely that (1) Chinese medicine compounds are commonly comprised of primary, secondary and adjuvant drugs, and some components of Chinese medicine are absorbed in the blood stream and become active after metabolism, so administration of the Chinese medicine compound often results in treatment effects in the whole body. (2) Monomers of Chinese medicine compounds interact synergistically, exhibiting superior effects over a lone monomer. (3) Some actual effective monomers may not have been found in the present study.

Ginseng has been reported to promote icariin absorption and distribution, delay elimination, and effectively improve icariin bioavailability^[14]. The major active component of ginseng is ginsenoside, and the major component of *Panax notoginseng saponins* also contains the ginsenoside Rg1. A previous study indicated that icariin plus *Panax notoginseng saponins* can reduce acetylcholinesterase activity in the brain tissues of beta-amyloid (25–35)-treated rats, improve learning, and inhibit the memory reduction induced by lateral ventricle injection of beta-amyloid (25–35)^[15]. Icariin plus *Panax notoginseng saponins* can also attenuate oxidative stress injury induced by beta-amyloid (25–35) in the hippocampal CA1 of rats and improve their learning and memory^[16]. The present study established an *in vitro* Alzheimer's disease

cell model and determined cell viability and the extent of early apoptosis using an MTT assay and Annexin V-fluorescein isothiocyanate/propidium iodide staining.



Results from both methods showed that the combination of monomers of icariin and *Panax notoginseng saponins* exerted similar effects to *Yizhijiannao* Granule with regard to the inhibition of beta-amyloid (25–35)-induced early PC12 apoptosis and the protection of PC12 cells from beta-amyloid (25–35)-induced injury, indicating synergistic effects of icariin and *Panax notoginseng saponins*. Icariin plus *Panax notoginseng saponins* may have multiple-layer and multiple-target effects in Alzheimer's disease treatment. The present study provides a reference for the development of drugs

to substitute for *Yizhijiannao* Granule for the prevention and treatment of Alzheimer's disease.

MATERIALS AND METHODS

Design

A comparative observation and *in vitro* cytology study.

Time and setting

The experiment was performed in the Laboratory of Neurobiology, Xiangya School of Medicine, Central South University, China from May 2010 to March 2011.

Materials

Animals

A total of 40 healthy male Sprague-Dawley rats, aged 3 months, weighing 300 ± 20 g, were purchased from the Department of Laboratory Animal Science, Xiangya School of Medicine, Central South University and housed in the Department of Laboratory Animal Science, Xiangya School of Medicine, Central South University. Animal procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China^[17].

Drugs

Yizhijiannao Granule was composed of 15 g *Yinyanghuo*, 15 g *Suoyang*, 10 g *Sanqi*, 10 g *Ciwujia*, 10 g *Radix Dipsaci* from Sichuan, China, 10 g *Baiziren* and 5 g *Shuizhi*. The crude drugs were purchased from the Dispensary of traditional Chinese medicine, Xiangya Third Hospital of Central South University, China, and identified by the Laboratory of Chinese Medicine Pharmacology, Xiangya Third Hospital of Central South University. All components were mixed, water-extracted, condensed, and dried to prepare a dry extract. Each kind of Chinese medicine was prepared as 25 g granules, and each milliliter of concentrated solution (from which water was extracted) contained 3 g of crude drugs. The icariin, catechin, *Panax notoginseng saponins* and eleutheroside E content was controlled using high-performance liquid chromatography according to the *Chinese Pharmacopoeia*^[18]. During drug preparation, the quality of the drugs was strictly monitored by Hunan Dekang Pharmaceutical Co., Ltd., Hunan, China.

Monomers of icariin, catechin, *Panax notoginseng saponins* and eleutheroside E were purchased from Beijing Heng Yuan Qi Tian Institute of Chemical Technology, China, with the purity of all drugs > 98%.

Cell line

Undifferentiated PC12 cells (a rat adrenal gland

pheochromocytoma cell line with characteristics typical of nerve cells) were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Science.

Methods

Preparation of beta-amyloid (25–35)

beta-amyloid (25–35) (Sigma, St. Louis, MO, USA) was dissolved in PBS to a concentration of 250 μ M, incubated at 37°C for 4 days until aggregated, and stored at –80°C.

Establishment of the Alzheimer's disease cell model

The Alzheimer's disease cell model was established as previously described^[19-20]. Briefly, PC12 cells were incubated with 20 μ M beta-amyloid (25–35) for 24 hours. PC12 cells were seeded in a 24-well plate. Cell morphology was observed with an inverted phase contrast microscope (Nikon, Tokyo, Japan).

Preparation of Yizhijiannao Granule-containing serum

The 40 3-month-old male Sprague Dawley rats were randomly assigned to Yizhijiannao Granule-containing serum and blank serum groups, with 20 animals in each group. The rats in the Yizhijiannao Granule-containing serum group were intragastrically perfused with Yizhijiannao Granule-containing serum at a dose according to the body surface area formula^[21], 4.5 g/d each. The blank serum group was intragastrically perfused with an equal volume of normal saline. Both groups were perfused twice daily for 5 consecutive days. Blood was harvested from the femoral artery 1 hour following the final perfusion, and serum was isolated in a sterile manner, filtered, and sterilized.

Screening for the effective concentration of the four monomers

Four concentrations of each monomer were used: 100, 10, 1, and 0.1 mg/mL (icariin, catechin, and eleutheroside E) or 500, 50, 5, and 0.5 mg/mL (*Panax notoginseng saponins*). The absorbance value of each well was determined after PC12 cells were cultured with each monomer for 24 hours using an MTT assay^[22].

Drug treatment of beta-amyloid (25–35)-treated PC12 cells

PC12 cells were cultured in a culture flask and trituated when the cells grew into a single layer. The concentration was adjusted to 10⁵ cells/mL and seeded in a 96-well plate, with 100 μ L in each well. Normal group cells were cultured in Ham's F-12 medium (Hyclone, Logan, Utah, USA) containing 5% fetal bovine serum (Gibco, Gaithersburg, MD, USA) and 15% horse serum (Hyclone). The cells in the Yizhijiannao Granule-containing serum,

Chinese medicine monomer and NGF groups were treated with the corresponding drugs while in the logarithmic phase. The screening concentrations mentioned previously were used, with five parallel wells at each concentration; 10 mg/L NGF (Promega, Madison, WI, USA) was added to the NGF group for 2 hours. Each group was treated with beta-amyloid (25–35) for an additional 24 hours. Cell viability was determined using an MTT assay^[22], and the early apoptosis rate was detected using flow cytometry (Becton Dickinson, San Jose, CA, USA).

Detection of PC12 cell apoptosis using Annexin V-fluorescein isothiocyanate/propidium iodide staining flow cytometry

The early apoptosis rate of PC12 cells was detected using Annexin V-fluorescein isothiocyanate/propidium iodide staining flow cytometry (Becton Dickinson) according to the Annexin V-fluorescein isothiocyanate kit (Bender Med Systems, Vienna, Austria). Cells from each group were collected, rinsed with PBS, centrifuged, washed once with diluted binding buffer solution (20 mM Tris, 250 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.5% NP-40 and protease inhibitor, diluted 5 times), and the cell concentration was adjusted to 1 \times 10⁶/mL using the above buffer solution, mixed evenly with 5 μ L diluted Annexin V-fluorescein isothiocyanate and 10 μ L propidium iodide, incubated in the dark for 10 minutes and detected by flow cytometry. Cells were collected using Cell Quest software (Becton Dickinson) and 1 \times 10⁵ cells from each sample were detected. Results were represented as the percentage of positive cells (%). Data were analyzed using Cell Quest Plot software. A scatter plot was made with fluorescein isothiocyanate and propidium iodide fluorescence intensity as parameters.

Statistical analysis

Measurement data were expressed as mean \pm SD and analyzed using SPSS 15.0 software (SPSS, Chicago, IL, USA). Intergroup means were compared using one-way analysis of variance, and paired comparisons of intergroup data differences were conducted using a Student-Newman-Keuls test. The alpha level was $\alpha = 0.05$.

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Author contributions: Ting Zhang conceived and designed the study, analyzed data, and wrote the manuscript. Zhanwei Zhang integrated and provided experimental data. Keli Dong

revised the manuscript and guided the study. Guangcheng Li provided technical support and guided the study. Hong Zhu analyzed experimental data.

Conflicts of interest: None declared.

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