Compound *Danshen* tablets downregulate amyloid protein precursor mRNA expression in a transgenic cell model of Alzheimer’s disease

**Effects and a comparison with donepezil**

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**Abstract**

After gene mutation, the pcDNA3.1/APP595/596 plasmid was transfected into HEK293 cells to establish a cell model of Alzheimer’s disease. The cell model was treated with donepezil or compound *Danshen* tablets after culture for 72 hours. Reverse transcription-PCR showed that the mRNA expression of amyloid protein precursor was decreased in all groups following culture for 24 hours, and that there was no significant difference in the amount of decrease between donepezil and compound *Danshen* tablets. Our results suggest that compound *Danshen* tablets can reduce expression of the mRNA for amyloid protein precursor in a transgenic cell model of Alzheimer’s disease, with similar effects to donepezil.

**Key Words:** amyloid protein precursor; Alzheimer’s disease; transgenic cell model; compound *Danshen* tablets; Chinese medicine; neural regeneration

**Abbreviations:** AD, Alzheimer’s disease; APP, amyloid protein precursor; Aβ, amyloid β protein; Aβ, amyloid β protein

**INTRODUCTION**

Alzheimer’s disease (AD) is pathologically characterized by senile plaques, neurofibrillary tangles and death of nerve cells, in addition to diffuse cortical atrophy. The well-accepted amyloid β protein (Aβ) hypothesis assumes that abnormally increased Aβ is the main cause of senile plaque formation. The amyloid cascade reaction induced by Aβ fragment aggregation and deposition is an important process in AD pathology. Aβ can produce neurotoxicity through direct or indirect pathways, resulting in neuronal degeneration or death and learning and memory impairment. Under normal culture conditions in AD cell models, the mRNA for amyloid protein precursor (APP) is present at high levels. The translated APP proteins can be converted to Aβ, leading to cell toxicity.

Compound *Danshen* tablets are composed of *Radix Salviae Miltiorrhizae*, *Radix Notoginseng*, and *Borneol Syntheticum*. They can improve circulation in cardio-cerebral vessels. The main components include tanshinone, salvianolic acid, A, B, C, notoginsenoside R₁, ginsenoside Rb₁, ginsenoside Rg₁, and ginsenoside Rd. In a previous study from our group, compound *Danshen* tablets were shown to prevent and ameliorate AD-like symptoms in AD rats, improving their spatial cognitive impairment, reducing excitatory amino acid glutamic acid content and inhibiting Aβ production[1]. Recent evidence indicates that compound *Danshen* tablets can enhance vascular growth factor expression around the infarction in rats with acute brain ischemia and that it can promote nerve cell repair[2]. Tanshinone can inhibit excess Aβ deposition and attenuate Aβ-induced neurotoxicity.

In the present study, an APP transgenic AD cell model was treated with compound *Danshen* tablets and donepezil to investigate the effects of the tablets on APP expression, and compare these effects with those of donepezil.

**RESULTS**

**Serum total RNA purity analysis**

Sprague-Dawley rats were intragastrically perfused with donepezil and compound *Danshen* tablets to prepare sera containing...
each treatment. Groupings are shown in Table 1. Total RNA was extracted from AD model cells using the one-step guanidinium thiocyanate method and the purity of extracted RNA was determined. As shown in Table 1, the absorbance ratio of 260 nm to 280 nm for the RNA from each group ranged from 1.6 to 2.1, indicating high purity of the RNA extracted from each group.

Table 1  Total RNA determination results  (x±s, n=5)

| Group                      | A_{260} nm | A_{280} nm | A_{260 nm/280 nm} |
|----------------------------|------------|------------|-------------------|
| CDT-containing serum       | 0.171±0.005| 0.107±0.008| 1.603±0.008       |
| Blank serum                | 0.154±0.009| 0.074±0.004| 1.903±0.005       |
| Donepezil-containing serum | 0.146±0.007| 0.086±0.007| 1.890±0.009       |
| CDT (24 hours)             |            |            |                   |
| Blank serum                | 0.170±0.008| 0.084±0.007| 2.033±0.007       |
| Donepezil-containing serum | 0.151±0.009| 0.079±0.006| 1.901±0.008       |
| CDT (48 hours)             |            |            |                   |
| Blank serum                | 0.189±0.005| 0.097±0.008| 1.942±0.006       |
| Donepezil-containing serum | 0.157±0.003| 0.087±0.007| 1.803±0.005       |
| CDT (72 hours)             |            |            |                   |

Results are expressed as mean±SD of five rats in each group. The dose of donepezil-containing serum was 1.5 mg/kg; the doses of CDT-containing serum were 0.5, 1.0, 2.0, and 4.0 mg/kg. CDT: Compound Danshen tablets; CDT: culture time following administration; A: absorbance.

Effects of compound serum containing compound Danshen on APP mRNA expression in an AD cell model

After being cultured for 72 hours, APP transgenic cells were treated with normal rat serum (blank serum group), donepezil-containing serum × 1 (donepezil 5.8 mg/kg), and compound Danshen tablets at 157.5, 315.0, 630.0, and 1 260.0 mg/kg, to prepare compound Danshen tablets-containing serum × 0.5, 1.0, 2.0, and 4.0, respectively.

Following electrophoresis, APP reverse transcription-PCR products were observed under an ultraviolet lamp (Figure 1). PCR product agarose gel electrophoresis was scanned and data were analyzed using Kodak 2.0 software (Table 2). The results showed that APP mRNA expression was higher in cells treated with sera containing donepezil and compound Danshen compared with those treated with blank serum (P<0.01, P<0.05). However, the level of APP mRNA expression was lower in cells treated with sera containing compound Danshen and donepezil compared with the blank serum group after culture for 24 and 48 hours (P<0.01). No significant difference was found between the donepezil and compound Danshen tablet groups (P>0.05).

![Figure 1](https://example.com/image1.png)

**Figure 1** Amyloid protein precursor (APP) mRNA expression in the transgenic cell model of Alzheimer’s disease following compound Danshen tablet treatment. Lanes 1–6: Blank, cells treated with sera containing compound Danshen × 0.5, 1.0, 2.0, and 4.0 and donepezil following administration for 24 hours; Lanes 7–12: Blank, cells treated with sera containing compound Danshen × 0.5, 1.0, 2.0, and 4.0 and donepezil following administration for 48 hours; Lanes 13–18: Blank, cells treated with sera containing compound Danshen × 0.5, 1.0, 2.0, and 4.0 and donepezil following administration for 72 hours.

| Group                  | Administration time (hour) | 24 | 48 | 72 |
|------------------------|---------------------------|----|----|----|
| Blank serum            | 0.869±0.065               | 0.895±0.044 | 0.916±0.059 |
| Donepezil-containing serum | 0.653±0.046                | 0.802±0.057 | 0.869±0.053 |
| CDT-containing serum   | 0.476±0.072                | 0.579±0.076 | 0.856±0.069 |
| CDT: Compound Danshen tablets | 0.598±0.059             | 0.708±0.051 | 0.863±0.051 |
| Donepezil-containing serum | 0.602±0.055                | 0.797±0.034 | 0.869±0.047 |
| Donepezil-containing serum | 0.487±0.067                | 0.649±0.050 | 0.859±0.062 |

*P<0.05, **P<0.01, vs. blank serum group. Results are expressed as mean±SD of five rats in each group. The gray scale ratio of β-amyloid protein precursor gene to β-actin was used to represent relative β-amyloid protein precursor mRNA expression. Comparisons of means among groups were performed using one-way analysis of variance; paired comparisons of means among groups were performed using the least significant difference t-test. CDT: Compound Danshen tablets.

DISCUSSION

The HEK293 cell strain is derived from human embryonic kidney epithelial cells. It has been frequently used in
transfection experiments owing to their high efficiency for transfection and culture. Stably transfected HEK293 cells can express secreted proteins or membrane proteins within or outside the cells. In the present study, we transfected HEK293 cells with pcDNA3.1/APP595/596 plasmid to construct an AD cell model with overexpression of Aβ protein. A previous study from our group showed that various components in compound Danshen tablets can intervene in AD processes through different pathways. For example, tanshinone significantly improves learning and memory impairment in rats following intrahippocampal injection of Aβ, protects the cholinergic system in AD-like rats, regulates nitric oxide synthase expression and antagonizes oxidation and inflammation[9,10]. These results suggest that tanshinone might inhibit excess Aβ deposition and attenuate Aβ neurotoxicity. Tanshinone IIA can increase cortical and hippocampal glutamic acid and gamma-aminobutyric acid content in mice with vascular dementia, decrease malondialdehyde production, increase superoxide dismutase and glutathione peroxidase activities[10], and inhibit apoptosis of nerve cells[11]. In the present study, the APP mRNA level was high in our cell model. Danshen can significantly improve spatial cognitive impairments in rats with unilateral temporal lobe ischemic damage[7]; salvianolic acid can ameliorate brain ischemia and inhibit glumatic acid release[8]. The main component of Radix Notoginseng, triterpenoid saponin, is similar to ginsenoside. Modern studies have shown that panax notoginseng fraction B can upregulate muscarinic receptor density[9], inhibit Aβ production and improve hippocampal acetylcholine release[10]. Panax notoginseng saponins can attenuate Aβ-induced cytotoxicity and promote cell process growth, thereby antagonizing the progression of senile dementia[11]. Panaxsaponin exhibits evident protection for learning and memory injury[12-13]. Broneolum synthenticum is used as an adjuvant drug in this prescription; it can induce resuscitation, activate consciousness, improve blood-brain barrier permeability, increase drug concentrations in the brain and enhance drug neuropharmacologic actions[14]. A previous study showed that compound Danshen tablets significantly improve learning and memory function in experimental AD rats and inhibit Aβ production in the brain[15]. In the present study, pretreatment of cells with sera containing compound Danshen significantly reduced APP mRNA expression in AD cells and decreased APP and Aβ protein synthesis. The dose-effect of Chinese medicine, especially compounds, is not obvious. According to serum pharmacology, 7-day administration can enable steady plasma-drug concentrations to be reached. However, the differences among doses remain poorly understood. Further studies should utilize high performance liquid chromatography for serum detection. In addition, the present study was performed at the gene level, so future studies should focus on protein levels.

The results of the present study show that compound Danshen tablets can downregulate APP mRNA expression in a cell model of AD, possibly attenuating AD progression. Further randomized, double-blind, multicenter clinical studies are needed to verify these results.
compound *Danshen* tablets for one rat was 315.0 mg/kg and that for donepezil was 5.8 mg/kg. The 30 rats were randomly assigned to a blank serum group and five drug-containing serum groups, with five animals in each group. Donepezil was used as positive control (cholinphospholipid enzyme inhibitor) \( \times 1 \) (5.8 mg/kg). The concentrations of compound *Danshen* in serum \( \times 0.5, 1, 2, \) and 4 were 157.5, 315.0, 630.0, and 1 260.0 mg/kg, respectively; rats in the blank serum group were given the same volume of normal saline, once a day for 7 consecutive days, so no compound *Danshen* was present in their sera. Blood was extracted from the abdominal aorta 1 hour after the final administration, and sera were isolated. Complement was deactivated at 56°C for 30 minutes. Serum was filtered through a microporous membrane (Jinteng, Tianjin, China), packaged, and stored at -20°C.

**Extraction and verification of pcDNA3.1/APP595/596 plasmid**

pcDNA3.1 APP plasmid was extracted according to a previously described method\(^\text{[16]}\) and verified by Shanghai Sangon, China. Sequencing results showed that, in addition to the mutations within amino acid codons 595 and 596 (described previously), there was an unexpected mutation at the fourth basic set (C→G at position 2 086 bp in the Appc DNA sequence), which would influence amino acid expression, Leu→Val.

**HEK293 cell culture**

HEK293 cells were cultured in high-glucose Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum in 5% CO\(_2\), under saturated humidity at 37°C, and trypsinized (0.25%). Cells adhered like epithelial cells. Cells in the logarithmic phase were selected for experiments.

**Establishment of AD transgenic cell model**

pcDNA3.1/APP595/596 plasmid was transfected into HEK293 cells using calcium acid phosphate transfection\(^\text{[16]}\). Briefly, 1 \( \mu \)g of DNA and 10 \( \mu \)L of CaCl\(_2\) (2.5 M), made to a 100- \( \mu \)L solution with sterile water was mixed with 100 \( \mu \)L of 2× PBS (pH 6.95) and stored at room temperature for 20–30 minutes, then mixed evenly every 10 minutes. The above solution was slowly aliquoted into the wells of a 6-well plate, mixed and left at room temperature for 30–50 minutes, before the addition of culture medium containing 10% fetal bovine serum. Cells were cultured in 5% CO\(_2\) at 37°C. The culture medium was replaced at 2 days, washed with serum-free Dulbecco’s modified Eagle’s medium, twice, and cultured with culture medium containing 10% fetal bovine serum. After 3 days, selective culture medium containing 800 \( \mu \)g/mL G418 was used to screen stably transfected clones. After 10–14 days, the clones were screened using G418. Clonal cells were transferred and expanded.

**Interventions**

After the transgenic AD cell model had been in culture for 72 hours, the blank serum group was treated with normal rat serum; the donepezil group was treated with donepezil-containing serum \( \times 1 \) (5.8 mg/kg); and the compound *Danshen* tablet groups were treated with sera containing compound *Danshen* \( \times 0.5, 1, 2, \) and 4, followed by additional culture for 24, 48 and 72 hours for all groups.

**Reverse transcription-PCR for APP mRNA expression in the AD transgenic cell model**

APP mRNA expression levels were determined using reverse transcription-PCR and PCR primer sequence is shown as follows:

| Gene | Sequence |
|------|----------|
| APP  | Forward: 5′-GCC AAG AAG TCT ACC CTG-3′  
Reversed: 5′-AAC TCT ACC CCT CGG AAC-3′ |

cDNA synthesis:

| Reagent | Volume (\( \mu \)L) |
|---------|-------------------|
| RNA     | 5 \( \mu \)L |
| Oligo(dT)\(_{16}\) | 0.5 \( \mu \)g |
| Rnasin  | 20 U |
| 5 × RT buffer | 4 \( \mu \)L |
| 10 mM dNTPs | 1 \( \mu \)L |
| AMV reverse transcriptase | 10 \( \mu \)L |
| Supplemented with H\(_2\)O to | 20 \( \mu \)L |

The solution was placed in a 1.5-mL Eppendorf centrifuge tube, stored at 42°C for 60 minutes, cooled on ice, centrifuged for seconds, and subjected to PCR. The PCR system was constructed using 0.5-mL sterile micro centrifuge tubes.

**PCR system for amplifying target gene:**

| Reagent | Volume (\( \mu \)L) |
|---------|-------------------|
| Template | 2 |
| 10 × PCR buffer | 2 |
| 10 × dNTPs | 2 |
| P1 | 0.5 |
| P2 | 0.5 |
| TaqE | 0.2 |
| H\(_2\)O | 13 |

The centrifuge tubes were placed in a thermal cycler. PCR amplification was performed using the T-Gradient PCR system (Whatman Biometra, Germany). PCR conditions were as follows:

| Conditions | Time (s) |
|------------|---------|
| 94°C        | 2 minutes |
| 94°C        | 30 seconds |
| 58°C        | 30 seconds |
| 72°C        | 30 seconds |
| 72°C        | 10 minutes |
| 4°C         | ∞ |

30 cycles

PCR products were placed on ice. Products (10 \( \mu \)L) were electrophoresed in 1% agarose gels containing 0.5 \( \mu \)g/mL ethidium bromide for detection. Results were analyzed using Kodak 2.0 software (Rochester, NY, USA). The gray scale ratio of APP gene to APP was
used to represent relative APP mRNA expression.

**Statistical analysis**

Measurement data are expressed as means ± SD. Data were analyzed using SPSS version 17.0 software (SPSS, Chicago, IL, USA). Data were subjected to tests of normality and normal distribution. Comparisons of means among groups were performed using one-way analysis of variance; paired comparisons of means among groups were performed using the least significant difference- t test. For heterogeneity of variance, Dunnettt’s T3 method was used. A value of \( P < 0.05 \) was considered to represent statistical significance.

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**Author contributions:** Ren’an Qin was in charge of obtaining funds, provided technical and data analysis support, guided the study, and revised the manuscript. Jiajun Wang conducted the molecular biology experiments, analyzed the data, conducted the statistical analysis and wrote the first draft of the manuscript. Hua Hu conceived and designed the study. Desheng Zhou revised the manuscript and guided the study. Yang Yang, Xiaoxuan Yao, and Xiaopeng Sun assisted with the experiments.

**Conflicts of interest:** None declared.

**Ethical approval:** This study received permission from the Animal Ethics Committee of Hunan University of Traditional Chinese Medicine, China.

**REFERENCES**

[1] Qin RA, Luo JB, Huang ZY, et al. Effect of compound danshen tablets on the disturbance in learning and memorizing and expression of brain β-amyloid precursor protein in rat Alzheimer’s disease model. Zhonghua Zhongyi Yao Zazhi. 2005;6(20):377-378.

[2] Qin RA, Zhang CG, Hu H, et al. Effect of compound danshen tablet on rats with acute cerebral ischemia. Zhongfeng yu Shenjing Jibing Zazhi. 2011;9(9):1092-1094.

[3] Liu YH, Li J. Protective effects of salviol on aphrenia in mice induced by amyloid beta-protein and its mechanisms. Zhongfeng yu Shenjing Jibing Zazhi. 2007;24(1):64-66.

[4] Li L, Chen ZY, Ru LQ. Mechanism of tanshineone on composite dementia model rats induced by D-galactose combination and Aβ1-40. Lanzhou Daxue Xuebao. 2010;2(36):12-16.

[5] He Zhi, Pan ZH, Lu WH. Neuroprotective effects of tanshineone ia on vascular dementia in rats. Shizhen Guoyi Guoyao. 2009;20(12):3022-3024.

[6] Yu XQ, Xue CC, Zhou ZW, et al. Tanshineone iiB, a primary active constituent from Salvia miltiorrhiza, exerts neuroprotective effect via inhibition of neuronal apoptosis in vitro. Phytother Res. 2008;22(6):846-895.

[7] Jiang SJ, Wu WP, Kuang PG, et al. Radix salviae miltiorrhizae (RSM) could improves spatial cognition of rats with left cerebral temporal infarction and expression of HSP32. Zhongfeng yu Shenjing Jibing Zazhi. 1999;16(5):257-259.

[8] Wang J, Zhang JT. Anti cerebral ischaemia action and inhibitory effect on glutamate release of total salvianolic acid. Zhongguo Yaolixue yu Duluixe Zazhi. 1999;13(3):197-199.

[9] Sun QX, Li Y, Hu YE, et al. Effects of panax notoginseng fraction B on stimulant demential model in SD rats. Zhongguo Linchuang Kangfu. 2004;8(28):6152-6154.

[10] Guo CJ, Wu JX, Li RX. The effects of PNS on Alzheimer’s disease model and mechanism of the effects. Zhongguo Yaofang. 2004;15(10):598-600.

[11] Lu ZP, Wang NP, Zhong ZG. Protection of panaotoginsengsaponins against NG108-15 cells in senile dementia model induced by amyloid beta-peptide 25-35. Zhongguo Linchuang Kangfu. 2004;8(34):7876-7883.

[12] Zhang Q, Liu A, Zhou Y, et al. Panax ginseng ginsenoside Rg1 Protects memory impairment via anti-apoptosis in a rat model with vascular dementia. J Ethnopharmacol. 2008;115(3):441-448.

[13] Wang YZ, Wang YS, Chu SF, et al. Nootropic signal transduction of ginsenoside Rg1. Zhongguo Yaolixue Tongbao. 2008;24(6):740-743.

[14] Liu Na, Gao XC. The study of blood-brain barrier opening by borneol. Welfang Yixueyuan Xuebao. 2007;29(5):398-400.

[15] Qin RA, Luo JB, Chen M, et al. Effect of compound Danshen Tablets on amino acid neurotransmitters in brain of Alzheimers disease rats. Zhong Cao Yao. 2004;35(8):905-907.

[16] The Ministry of Science and Technology of the People’s Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006-09-30.

[17] Liu JW. The Methodology of Pharmacological Experiment. New Technology and Method. Beijing: Chemical Industry Press. 2003.

[18] Feng L. Research with QDs-SA Fluorescence for APP and Aβ proteins of Alzheimer’s disease molecular imaging. Hunan: Central South University. 2009.

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