Effects of Ginseng Protein on Gut Microbiota and BDNF/TrkB Signaling Pathway in Alzheimer's Disease Mice

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Abstract [**Objectives**] To investigate the effects of ginseng protein on gut microbiota and BDNF/TrkB signaling pathway in Alzheimer's disease (AD) mice. [**Methods**] D-galactose/$AlCl_3$ co-induction was used to establish AD model, and mice were randomly divided into normal group 1, normal group 2, model group 1, model group 2, ginseng protein group, and microbiota transplantation group. Morris water maze experiment was used to evaluate learning and memory ability, and Western blot method was used to detect the expression of APP, p-Tau, BDNF, TrkB, p-TrkB proteins in brain tissue, and 16S rDNA was used to detect diversity of fecal microbiota. [**Results**] Ginseng protein and microbiota transplantation can shorten the escape latency of mice ($P<0.05$), increase the number of crossing platforms ($P<0.05$), reduce the expression of APP and p-Tau proteins in brain tissue ($P<0.05$, $P<0.01$), increase the expression of BDNF, p-TrkB, p-TrkB/TrkB proteins ($P<0.05$, $P<0.01$), and reduce the abundance of *Alloprevotella*, *Ruminococcaceae*_UCG-014, *Prevotellaceae*_UCG-001, and *Ruminococcus*_1 ($P<0.05$, $P<0.01$). [**Conclusions**] The action mechanism of ginseng protein anti AD may be through regulating gut microbiota diversity and activating the BDNF/TrkB signaling pathway.

Key words Ginseng protein, AD, Gut microbiota, BDNF/TrkB signaling pathway, 16S rDNA

1 Introduction

Ginseng protein is one of the effective components of ginseng, and has *in vivo* and *in vitro* anti Alzheimer's disease (AD) effects[1-2]. The action mechanism is related to activating cAMP/cAMP-response element binding protein (cAMP/CREB) signaling pathway. Brain derived neurotrophic factor (BDNF) is downstream effect factor of CREB, could combine with tyrosine kinase receptor B (TrkB), and induce TrkB self phosphorylation increases, further promoting neuron growth, survival, and differentiation[3-4], with certain protective effects on neuronal damage induced by β-amyloid protein (Aβ)[5]. The BDNF/TrkB signaling pathway is closely related to cognitive function, and its inactivation may lead to abnormalities in cognitive function[6]. Meanwhile, BDNF expression is closely related to gut microbiota. Disturbance of gut microbiota will reduce the expression of BDNF in the cerebral cortex and hippocampus, resulting in dysfunction of the central nervous system, behavioral abnormalities, cognitive disorders and even AD[7]. It is reported that the abundance and diversity of gut microbiota in AD patients are significantly reduced[8]. This paper aimed to explore the anti AD mechanism of ginseng protein through 16S rDNA microbial diversity sequencing, gut microbiota transplantation combining with Morris water maze, and Western blot experiments.

2 Materials and methods

2.1 Materials

2.1.1 Animals. 90 SPF level of KM mice, female, body weight of 30 - 35 g, bought from Liaoning Changsheng Bio-technology Co., Ltd. Production license number of experimental animals: SCXK (Liao)2015-0001.

2.1.2 Drugs, reagents and instruments. Ginseng was purchased from the Ginseng Planting Base of Jilin Agricultural University and has been certified as authentic by Professor Jiang Dacheng from the Traditional Chinese Medicine Appraisal Teaching and Research Office of Changchun University of Traditional Chinese Medicine. Amyloid precursor protein (APP, item number: A16265), BDNF (item number: A11028), TrkB antibody (item number: A12325) (Wuhan ABclonal Biotech Co., Ltd.); phosphorylated microtubule-associated protein (p-Tau, phosphorylation site: ser 396, item number: AF3418), phosphorylated tyrosine kinase B (p-TrkB, phosphorylation site: Tyr515, item number: AF3462) antibody (USA Affinity Company); HRP labeled goat anti rabbit IgG (H + L) (item number: WLA023a) (Shenyang Wanlei Biotechnology Co., Ltd.). MS-1 type of Morris water maze video tracking system (Chengdu Taimeng Technology Co., Ltd.); electrophoresis instrument, semi dry film transfer instrument (USA Bio-Rad Company); ultra sensitive chemiluminescence imaging system (Shanghai Qinxiang Scientific Instrument Co., Ltd.).

2.2 Methods

2.2.1 Preparation of ginseng protein. After crushing ginseng, 10

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times of PBS buffer solution (pH 7.5) was added to soak. It was extracted twice after overnight at 4 ℃. The extract was centrifuged at 5 000 r/min for 30 min at 4 ℃. The supernatant was combined, filtered, and concentrated, and 20 mL of concentrated solution was taken for 0.45 μm of microporous membrane filtration. After washing and balancing with ultrapure water, it was injected in AKTA protein purification system for detection. The flow with UV absorption peak was taken, combined, frozen, and dried, and the yield was about 0.6%. Before use, ginseng protein freeze-dried powder was taken, and distilled water was added to prepare 0.01 g/mL of solution. It was refrigerated at 4 ℃, and the usage time of each formulation should not exceed 3 d.

2.2.2 Grouping and administration. 90 mice were randomly divided into normal group 1, normal group 2, model group 1, model group 2, ginseng protein group, and microbiota transplantation group, with 15 mice in each group. Except for two normal groups, the mice in the other groups were subcutaneously injected with D-galactose (140 mg/kg) and $AlCl_3$ (20 mg/kg) by gavage to establish an AD model, once a day for 60 consecutive days. Mice in normal group 1 were gavaged and injected with distilled water and physiological saline of the same volume as the model group every day. After 30 d of modeling, mice in the ginseng protein group were given the 0.1 g/kg of corresponding solution by gavage, once a day for 30 consecutive days. Dosage referred to the report of the literature[2]. After 60 d of modeling, mice in microbiota transplantation group were given 0.2 g/kg [about the equivalent dose of clinical human fecal bacterial fluid (1.67 g/kg[10])] of mouse fecal bacteria solution by gavage (the preparation method was to collect the feces of ginseng protein group mice after 30 d of administration in a sterile manner, and quickly mix with sterile saline at a ratio of 1∶5 and seal[9]. Three layers of gauze was used to filter and remove insoluble large particles. The filtrate was centrifuged at 4 ℃ and 3 000 r/min for 3 min, and the supernatant was discarded. It was resuspended with an equal amount of sterile saline, repeated twice, and stored at −80 ℃), once a day for 30 consecutive days. Then, normal group 2 and model group 2 mice with the same treatment time were used as blank and model control.

2.2.3 Morris water maze test. After 26 to 30 d of modeling, Morris water maze experiment was used to screen the mice in each group. The ones with larger dispersion were removed, and 10 mice were retained in each group. The subsequent experiment was divided into two stages. The first stage was the administration of ginseng protein for 25 − 30 d (namely modeling for 55 − 60 d), and the normal group 1, model group 1, and ginseng protein group mice were tested. The second stage involved microbiota transplantation for 25 − 30 d (namely modeling for 85 − 90 d), with the remaining mice undergoing testing. The first 5 d were positioning navigation experiments, with one training per day in each of the 4 quadrants for 60 s. The average score of the 4 quadrants on the 5th d was used as the positioning navigation experiment result. On the 6th d, the underwater platform was removed, and a space exploration experiment was conducted. The farthest quadrant from the platform was used as the entry point to evaluate the learning and memory abilities of mice by escape latency period (*i. e.* the time it takes to find the platform from the entry point) and times of crossing the platform after disembarking.

2.2.4 Expression of APP, p-Tau, BDNF, TrkB, p-TrkB, and p-TrkB/TrkB proteins detected by Western blot method. After Morris water maze test finished, each group of mice was decapitated and killed, and hippocampal tissue was taken. The same group was randomly merged into three samples in each group. After liquid nitrogen grinding and RIPA cracking liquid splitting (including 10 μL of PMSF), BCA protein quantitative assay kit was used to detect the protein concentration. After separation by 10% SDS-PAGE gel electrophoresis, half dry conversion was performed. The first antibody (APP, p-Tau, BDNF, TrkB, p-TrkB, 1∶1 000) and the second antibody (anti rabbit IgG, 1∶4 000) were incubated respectively, and ECL color rendering was developed. The chemiluminescence imaging system used GAPDH as the internal reference and ImageJ software to analyze the optical density value of stripe. In order to intuitively compare the difference in the improvement effect of ginseng protein and microbiota transplantation on pathological damage in mice, based on the reduction of APP or p-Tau protein expression in the model group 1 by ginseng protein, this paper converted the ginseng protein values and plotted them together with the microbiota transplantation group. The formula was: Ginseng protein group conversion = Ginseng protein group/(Model group 1 × Model group 2).

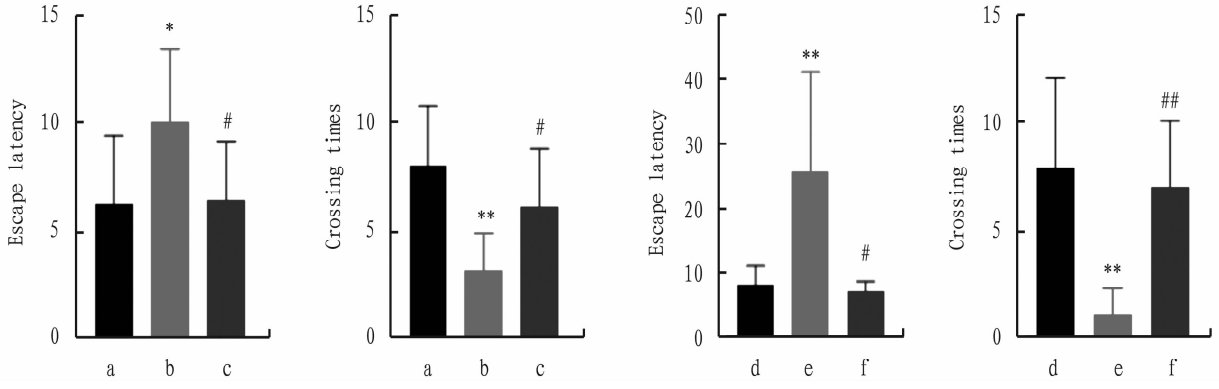
2.2.5 Analysis of gut microbiota diversity by 16S rDNA. After the Morris water maze experiment was completed for 1 h, the feces of normal group 1, model group 1, and ginseng protein group mice were collected aseptically, sealed and placed in a sterile EP tube, which was frozen at −80 ℃. The feces of 6 mice in each group (about 0.18 g each) were randomly selected, and the genomic DNA of the sample was extracted with DNA extraction kit. The DNA concentration was detected by agarose gel electrophoresis and NanoDrop2000. Taking genomic DNA as a template, specific primers with barcode and Tks Gflex DNA Polymerase were used for PCR amplification. After electrophoresis detection, the PCR products were purified with magnetic beads, and further PCR amplification was performed twice. Qubit quantification was performed on the purified PCR products. According to its concentration, equal amounts of samples were mixed, and sequencing on the machine was conducted. Using OTU (operational taxonomy unit, which divides sequences into many groups based on their similarity, one group is one OTU, and OTU classification is based on 97% sequence similarity), microbial community samples from different sources were analyzed at the phylum, class, family, genus, species, and other levels. In this paper, taking the genus

level as an example, the top ten in diversity species abundance ranking were selected for Boxplot analysis of relative abundance, and comparison of the dominant species within and between groups was conducted.

2.2.6 Statistical analysis. SPSS 25.0 software was used for processing, and the data was represented by ($\bar{x} \pm s$). Comparison among multiple groups was conducted using one-way ANOVA. $P<0.05$ indicated that the difference was statistically significant.

3 Results and analysis

3.1 Effect of ginseng protein on the learning and memory ability of mice After 60 d of modeling, compared with the normal group 1, the escape latency of mice in the model group 1 was prolonged, and the times they crossed the quadrant where the original platform was located was reduced ($P<0.05$, $P<0.01$). Compared with the model group 1, the ginseng protein group mice had a shorter escape latency and an increased crossing times ($P<0.05$). After 30 d of fecal bacterial transplantation (namely 90 d of modeling), compared with the normal group 2, the escape latency of mice in the model group 2 was prolonged, and the number of crossing platforms was reduced ($P<0.01$). Compared with the model group 2, the mice in the microbiota transplantation group had a shorter escape latency and an increase in the times they crossed the platform ($P<0.05$, $P<0.01$, Fig. 1).



Note: a. Normal group 1; b. Model group 1; c. Ginseng protein group; d. Normal group 2; e. Model group 2; f. Microbiota transplantation group. Compared with normal group1 and normal group 2, * shows $P<0.05$, and ** shows $P<0.01$. Compared with model group 1 and model group 2, # shows $P<0.05$, and ## shows $P<0.01$. The same below.

Fig. 1 Effect of ginseng protein on the learning and memory ability of mice ($\bar{x} \pm s$, $n=10$)

3.2 Effect of ginseng protein on the expression of APP and p-Tau proteins in mouse brain tissue Compared with normal group 1 and normal group 2, the expression of APP and p-Tau proteins in mouse brain tissue of model group 1 and model group 2 rose ($P<0.01$). Compared with model group 1 and model group 2, the expression of APP and p-Tau proteins in mice of ginseng protein group and microbiota transplantation group declined ($P<0.01$). Compared with normal group 2, the expression of APP and p-Tau proteins in mice brain tissue of model group 2 rose ($P<0.01$). Compared with model group 2, the expression of APP and p-Tau proteins in mice brain tissue of ginseng protein group and microbiota transplantation group declined ($P<0.05$, $P<0.01$). Compared with ginseng protein group, the expression of APP and p-Tau proteins in mice brain tissue of microbiota transplantation group had no significant difference ($P>0.05$, Fig. 2).

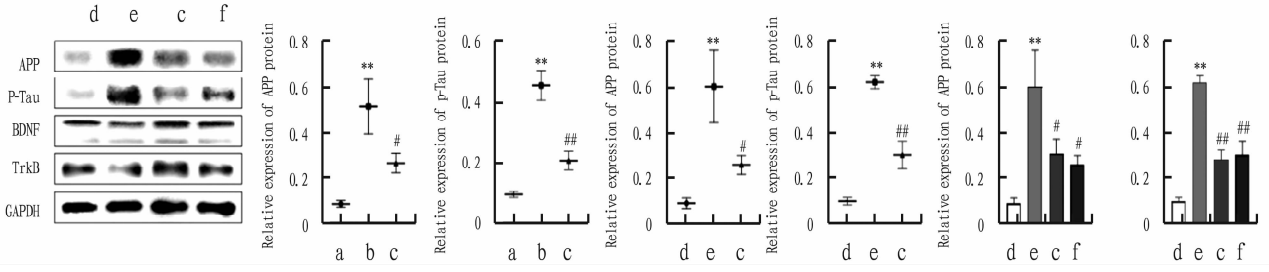


Fig. 2 Effect of ginseng protein on the expression of APP and p-Tau proteins in mouse brain tissue ($\bar{x} \pm s$, $n=3$)

3.3 Effect of ginseng protein on the expression of BDNF/TrkB pathway related proteins in mice Compared with normal group 2, the expression of BDNF, p-TrkB, p-TrkB/TrkB proteins in mice of model group 2 declined ($P<0.05$, $P<0.01$). Compared with model group 2, the expression of BDNF, p-TrkB, p-TrkB/TrkB proteins in ginseng protein group and microbiota transplantation group rose ($P<0.05$, $P<0.01$). Compared with ginseng protein group, TrkB protein expression in mice of microbiota transplantation group had no significant difference ($P>0.05$, Fig. 3).

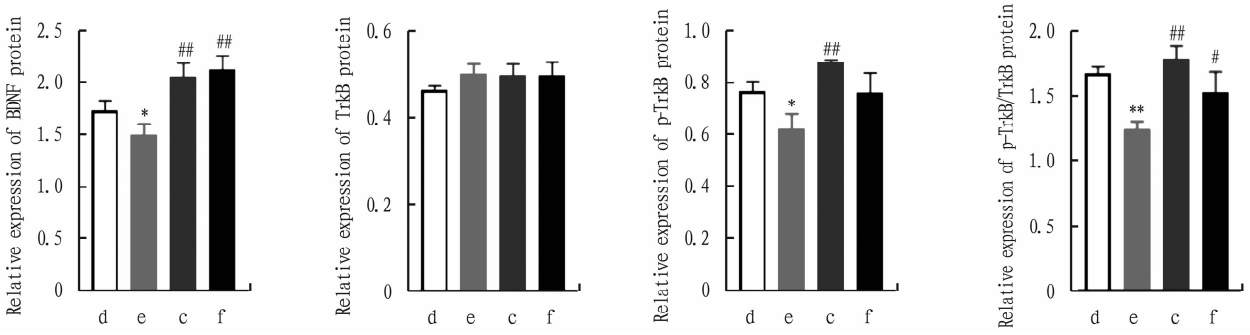


Fig. 3 Effect of ginseng protein on the expression of BDNF/TrkB pathway related proteins in mice ($\bar{x} \pm s$, $n = 3$)

3.4 Effect of ginseng protein on the diversity of gut microbiota in mice Compared with normal group 1, abundance of *Alloprevotella*, *Ruminococcaceae*_UCG-014, *Prevotellaceae*_UCG-001, *Rikenellaceae*_RC9_gut, and *Ruminococcus*_1 in differential bacteria genus of gut microbiota in model group 1 increased ($P < 0.05$, $P < 0.01$). Compared with model group 1, abundance of *Alloprevotella*, *Ruminococcaceae*_UCG-014, *Prevotellaceae*_UCG-001, and *Ruminococcus*_1 in differential bacteria genus of gut microbiota in ginseng protein decreased ($P < 0.05$, $P < 0.01$), while abundance of *Lachnospiraceae*_UCG-001 and *Desulfovibrio* increased ($P < 0.05$, $P < 0.01$, Fig. 4).

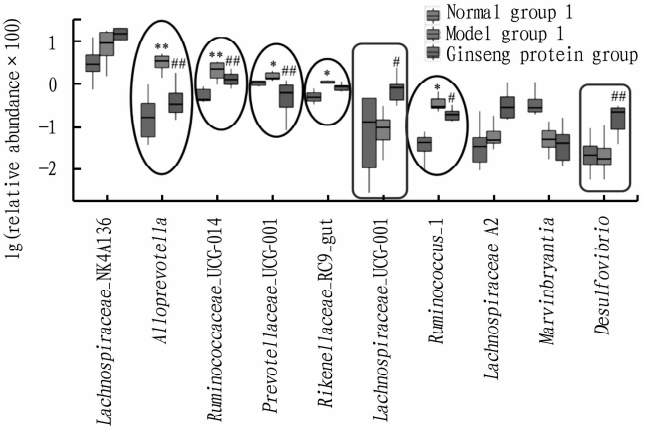


Fig. 4 Boxplot analysis of different species at the genus classification level in each sample ($n = 6$)

4 Discussion

Excessive production and insufficient clearance of Aβ are the initiating factors of AD onset[11]. Aβ is produced by hydrolysis of β amyloid precursor protein APP, and Aβ abnormal deposition will lead to damage of peripheral neurons and hyperphosphorylation of Tau protein to form neurofibrillary tangles[12], which will eventually lead to the occurrence of AD. In this paper, by studying the effects of ginseng protein and microbiota transplantation on the expression of APP and p-Tau proteins, the improvement of ginseng protein on the pathological damage of AD was explored. The results showed that ginseng protein and microbiota transplantation can inhibit the expression of APP and p-Tau proteins, and there was no difference between the two effects. It suggested that ginseng protein can inhibit the early process of AD, and regulating gut microbiota may be the main mechanism of its anti AD effect.

Morris water maze experiment is a widely used method for studying the spatial learning and memory abilities of rodents[10]. This paper found that ginseng protein and microbiota transplantation can significantly shorten the escape latency and the times of crossing the platform in AD model mice, suggesting that ginseng protein can improve the learning and memory ability of AD model mice, and this effect was related to the influence of gut microbiota. According to the reports, gut microbiota can affect brain function and regulate brain gene expression through the microbiota – gut – brain axis[14-15]. Gut microbiota and its metabolites are associated with central nervous degenerative diseases such as AD. Compared with the intestinal diversity of normal healthy people, the intestinal diversity of patients with AD is reduced[8]. The level of *Prevotellaceae* is increased[16], and the relative abundance of *Prevotellaceae*, *Ruminococcus* and *Rikenellaceae* is significantly different from the control group[17]. Through the difference analysis of OTU abundance at the level of genus classification, the results showed that ginseng protein can significantly affect the abundance of bacteria, make the number of bacteria close to that of the normal group, and reduce the abundance of *Alloprevotella*, *Ruminococcus*_1, and *Prevotellaceae*_UCG-001 related to AD. Since gut microbiota can regulate BDNF, synaptophysin and other nutritional factors or proteins that affect brain development and plasticity, and the previous research of the team also suggested that the anti AD effect of ginseng protein may be related to the activation of BDNF pathway, the influence of ginseng protein and microbiota transplantation on BDNF/TkB signaling pathway was further explored in this paper. The results showed that ginseng protein can activate the expression of BDNF and p-TrkB proteins, and increase the expression ratio of p-TrkB/TrkB protein, which was highly consistent with the effect of microbiota transplantation group. It suggested that regulating gut microbiota and activating BDNF/p-TrkB signaling pathway is one of the anti AD mechanisms of ginseng protein.

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(To page 79)

function, pulmonary ventilation and pulmonary exchange dysfunction. COPD patients are mainly manifested by long-term chronic cough and sputum production, often with acute exacerbations after cold and lung infection, it is a common respiratory disease affecting the health and quality of life of middle-aged and elderly people. Studies have pointed out that COPD patients need to be nursed for a long time and effectively to avoid affecting the risk factors of pulmonary function, and to strengthen pulmonary function exercise to improve their pulmonary function and quality of life[3]. Patients lack health expertise and are difficult to obtain significant self-care effects, and clinical nursing guidance is particularly important to help improve patients' pulmonary function and improve patients' quality of life.

The syndrome differentiation of TCM theory believes that COPD occurs due to the deficiency of the three organs of the lungs, spleen and kidneys, mainly lung and kidney qi deficiency, lung and spleen qi deficiency, lung and kidney qi yin, and TCM syndrome differentiation takes qi replenishment and yin as the main intervention method. This study implemented nursing intervention based on the syndrome differentiation of TCM, which significantly improved the pulmonary function and quality of life of patients compared with basic nursing intervention ($P<0.05$). TCM nursing based on syndrome differentiation dialectically implements dietary guidance, life intervention and emotions according to the type of patient's disease, has strong nursing pertinence, and can strengthen the improvement of patients' pulmonary function. The improvement of pulmonary function can effectively alleviate the clinical symptoms of patients, reduce the acute attack rate of patients, and then help improve the quality of life of patients. Based on syndrome differentiation of TCM, some scholars have implemented nursing interventions for patients with AECOPD, which is also effective in improving patients' pulmonary function and improving patients' quality of life[5-6].

In summary, TCM nursing based on syndrome differentiation in AECOPD patients can effectively improve the pulmonary function and quality of life of patients, and has significant clinical implementation value.

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(From page 68)

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