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EXPERIMENTAL STUDY

A combination extract of Renshen (*Panax Ginseng*), Yinyanghuo (*Herba Epimedii Brevicornus*), Yuanzhi (*Radix Palygalae*) and Jianghuang (*Rhizoma Curcumae Longae*) decreases glycogen synthase kinase 3β expression in brain cortex of APPV717I transgenic mice

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Abstract

OBJECTIVE: To investigate the neuroprotective mechanism of combination extract of Renshen (*Panax Ginseng*), Yinyanghuo (*Herba Epimedii Brevicornus*), Yuanzhi (*Radix Palygalae*) and Jianghuang (*Rhizoma Curcumae Longae*) (GEPT) in treating Alzheimer's disease on the target of glycogen synthase kinase 3β (GSK-3β).

METHODS: Three-month-old APPV717I transgenic mice were randomly divided into ten groups (*n*=12 per group) and intragastrically administrated vehicle or medicines: APP group was given 0.5% CMC, donepezil group was given donepezil (APP + D group) (0.92 mg/kg^{-1} · day^{-1}), and GEPT groups were given small dose of GEPT (APP+Gs group) (0.075 g/kg^{-1} · day^{-1}), medium dose (APP+Gm group) (0.15 g/kg^{-1} · day^{-1}), and large dose (APP+Gl group) (0.30 g/kg^{-1} · day^{-1}) for 4 or 8 months, respectively. Three-month-old C57BL/6J mice as vehicle controls (*n*=12) were given 0.5% CMC for 4 or 8 months as well. The GSK-3β expression in the cortex of 7- and 11-month-old APPV717I transgenic mice with and without GEPT or donepezil treatment and normal C57BL/6J mice were measured via Western blotting and Immunohistochemistry.

RESULTS: Immunohistochemistry analysis showed significant increase of GSK-3β in the cerebral cortex of 7-month-old APP group (compare to control group *P*=0.003), while the GSK-3β expression of donepezil or GEPT group were all significantly decreased (Donepezil vs APP: *P*=0.041; Gl vs APP: *P*=0.049; Gm vs APP: *P*=0.029; Gh vs APP: *P*=0.036). Western blot analysis showed similar results. The densitometric measures of GSK-3β in APP mice increased significantly as compared with the control group (*P*=0.008). And the GSK-3β expression in

donepezil and GEPT groups were all decreased. There was significant difference between Gh group or donepezil group and the control group (*P*=0.05). Similar findings were shown in the 11-month-old mice in each group, except for greater decrease of GSK-3β in the GEPT group.

CONCLUSION: GEPT can effectively decrease the level of GSK-3β expression in the brain cortex of APPV717I transgenic mice, and such effect is more significant in 11-month-old mice. This partially explains the neuroprotecting mechanism of GEPT in preventing and treating of AD.

Key words: Alzheimer disease; Mice, transgenic; Glycogen synthase kinase 3; Traditional Chinese Medicine; Chinese medical formula

INTRODUCTION

Alzheimer's disease (AD) is the most common progressive neurodegenerative disorder, affecting approximately 26 million patients worldwide up to 2006.[1] Neurofibrillary tangles, are composed of hyperphosphorylated microtubule-associated protein tau (MAPT), and senile plaques, accumulation of β-amyloid peptides, are two key neuropathological and diagnostic features of AD. While glycogen synthase kinase 3 (GSK-3), a 45-kDa, constitutively active, proline-directed serine/threonine kinase has garnered significant attention in the research of AD and other neurodegenerative diseases. It is expressed ubiquitously and found at high levels in the brain where it localizes predominantly to neurons, participates in a number of physiological processes, such as cell structure, metabolism, gene expression and apoptosis.[2, 3] GSK-3 also plays a pivotal and central role in the pathogenesis of AD. There was a 'GSK-3 hypothesis of AD' recently formulated[4] and it is a possible link between beta amyloid peptide and tau protein. In various cell culture, invertebrate and mammalian APPs of AD, over activity of GSK-3 leads to the hyperphosphorylation of tau, increased generation of amyloid-β (Aβ) and deficiency of learning and memory function.[5-7] More preciously, GSK-3β can phosphorylate numerous sites on tau and significantly impair its ability to bind microtubules. It may also play a role in regulating the degradation of tau[8] and the formation of insoluble tau fibrils[9]. Importantly, inhibiting GSK-3 activity can relieve the accumulation of Aβ peptides and the formation of hyperphosphorylated insoluble tau fibrils in the best available APPs of AD,[7,10-12] thus offers a new target to the treatment of AD.

As for the treatment of AD, a few fulfilled the ultimate disease-modifying goal. Neither cholinesterase inhibitors, nor the currently available N-methyl-d-aspartate receptor antagonist memantine are disease-modifying treatments. And the current evidences supporting the use of single herbs or herbal formulations is inconclusive or inadequate[13] and herbal mixtures may have advantages in treating AD with their multiple targets regulation.

GEPT, a combination of herbal extracts, also called GETO (Compound Jinsiwei) in our previous papers, consists of four active components from Renshen (*Panax Ginseng*), Yinyanghuo (*Herba Epimedii Brevicornus*), Yuanzhi (*Radix Palygalae*) and Jianghuang (*Rhizoma Curcumae Longae*).[14] Previous studies have proved that GEPT extract can markedly enhance learning and memory of AD rat APP induced by hippocampal injection of Aβ1-42 peptide or induced by intravenous injection of Aβ 1-40 peptide,[15] and reduce the level of Aβ in APPV717I transgenic mice via the inhibition of γ-secretase (presenilin-1) and the promotion of insulin degrading enzyme and neprilysin.[14] Moreover, GEPT also showed significant improvement on cognitive function in patients with amnestic mild cognitive impairment, an early stage of AD (*n*=101), consistently across different cognitive scales in a 24-week preliminary clinical study.[16] However, the GSK-3 inhibiting effects of GEPT in APP mice is still unknown. This study therefore will investigate the mechanism of GEPT on preventing and treating AD from the target of GSK-3β.

MATERIALS AND METHODS

Drugs preparation

GEPT was provided by Henan Wanxi Pharmaceutical Company Limited (Batch No.: 20010923) and dissolved in 0.5% carboxymethyl cellulose (CMC) at concentration of 30 mg/mL. Hydrochloric acid donepezil tablets were provided by Eisai (China) Pharmaceutical Company Limited (Batch No.: 090508A), crashed and dissolved into 0.5% CMC at concentration of 0.092 mg/mL.

Animals and medicine administration

Three-month-old APPV717I mice (C57BL/6J mice carrying mutated human APP-CT100 containing the London mutation V717I) and C57BL/6J mice (non-transgenic inbred mice, as vehicle controls), both half male and half female, were purchased from the Institute of Experimental Animals, Chinese Academy of Medical Sciences & Peking Union Medical College (Beijing, China). All animals were housed in the Pharmacological Experiment Center of Dongzhimen Hospital, Beijing University of Chinese Medicine, Beijing, China. They were maintained in a temperature-controlled (24℃) pathogen-free vivarium, on a 12:12 h light: dark cycle (12 h light: 6 am to 6 pm; 12 h dark: 6 pm to 6 am) with free access to food and water. All experimental procedures were performed under the requirements of the Provision and General Recommenda-

tions of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Research Ethics Board of Beijing University of Chinese Medicine.

Three-month-old APPV717I transgenic mice were randomly divided into ten groups (n=12 per group) and intragastrically administrated vehicle or medicines: APP group was given 0.5% CMC, donepezil group was given donepezil (APP + D group) (0.92 mg/kg^{-1} · day^{-1}), and GEPT groups were given small dose of GEPT (APP + Gs group) (0.075 g/ kg^{-1} · day^{-1}), medium dose (APP + Gm group) (0.15 g/ kg^{-1} · day^{-1}), and large dose (APP + Gl group) (0.30 g/ kg^{-1} · day^{-1}) for 4 or 8 months, respectively. Three-month-old C57BL/6J mice as vehicle controls (n=12) were given 0.5% CMC for 4 or 8 months as well.

Immunohistochemistry

All behaviorally-tested mice were deeply anesthetized with 10% chloral hydrate (40 mg/kg body weight, intraperitoneal), pericardially perfused with heparinized 0.9% saline, and removed the brain. The right hemispheres were immersion-fixed in 4% paraformaldehyde overnight at 4℃, and then processed in phosphate buffered saline (PBS) solution containing 30% sucrose. Seven days later, they were embedded in parafine. Serial coronal sections of the hippocampus were cut at 35 μm intervals for immunohistochemistry (IHC) staining. While the left hemisphere was snap frozen for Western blotting.

These brain sections were deparaffinised and degraded to distilled water. Antigens were unmasked in 0.01 M Citrate Buffer (Sun Biomedical Technology Co., Ltd., China) by microwave, and endogenous peroxidise activity was quenched by 0.3% hydrogen peroxide (Sun Biomedical Technology Co., Ltd., China) in methanol for 20 min at room temperature. Then the sections were blocked in 10% antibodies in 3% bull serum albumin/PBS for 30 min at 37℃. After excess serum was removed, sections were incubated with the primary antibody in humidified boxes at 4℃ overnight (GSK-3β 1∶500; blocking buffer was used as primary antibody in the internal control staining). They were washed once again and incubated with biotin conjugated secondary antibodies (1∶300; Fuzhou Maixin Ltd., China) at 37℃ for 30 min, and then washed again and incubated with Streptavidin-Biotin Complex (SABC) (Wuhan Boster Bioengineering Co., Ltd., China) for 1 h at 37℃. Subsequently, sections were developed using chromogen 3'3-diaminobenzidine tetrachloride, dehydrated, and affixed with coverslips. All brain sections chosen for staining were on a similar sagittal plane and contained approximately the same area of hippocampus. GSK-3β integrated optical density (IOD) was measured in immunostained sections, following the instructions of the Image Pro Plus 6.0 software (Media Cybernetics Company, MD, USA). "Nonspecific" immunological histological chemistry (IHC) staining in sections was chosen as the control area for comparison with the GSK-3β-immunopositive area in the neurons of the dentate gyrus.

Western blotting

Western blots were performed as described previously.[14] Briefly, snap-frozen brain tissues from hippocampus and cortex were weighted and homogenized with brain tissue lysis buffer (SABC) (Wuhan Boster Bioengineering Co., Ltd., China) at a ratio of 1∶10 (w/v) by a small pestle on ice for 2 min and incubated on ice for 30 min. Homogenates were centrifuged at 13 000 rpm at 4℃ for 30 min, and supernatants were collected. Protein level in the supernatants was determined by Bradford method[17] using Coomassie Brilliant Blue G-250 (Nanjing Jiancheng Bioengineering Institute, China). Loading buffer was added to samples at a ratio of 4∶1, after which they were placed in boiling water for 5 min and then chilled immediately on ice. 10 μL protein/well samples and 5 μL marker (10KD-170 KD) were loaded onto a 10% acrylamide gel (Sigma-Aldrich Co. LLC, St. Louis, USA) and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis using the Bio-Rad mini gel system (Bio-Rad, CA, USA). Proteins were then electro-blotted onto a polyvinylidine difluoride membrane. Membranes were blocked with 5% milk at 4℃ overnight, and then incubated with primary antibody (GSK-3β, ab131356, 1∶1000). After washing with PBS containing 0.5% Tween 20 (PBST) (Sun Biomedical Technology Co., Ltd., China), for 3 times, membranes were incubated at room temperature for 1 h with HRP-conjugated secondary antibody (Promega Co., WI, USA) at 1∶5000 dilutions on a shaker. Blots were developed using the luminol reagent (Pierce Biotechnology, Inc., France). Densitometric analysis of the blots was completed using the Phoretix 1D software (Total Lab Ltd., UK).

Statistical analysis

All data are analyzed by SPSS 13.0 software and presented as the mean±standard deviation (SD). One-way ANOVA was used when comparisons were made among those groups. P<0.05 was considered statistically significant.

RESULTS

GSK-3β expression levels in 7-month-old experimental mice

Immunohistochemistry analysis showed a significant increase of GSK-3β in the brain cortex of 7-months-old APPV717I transgenic mice as compared with the control group (P=0.003), while in the donepezil or GEPT treated transgenic mice were all significantly decreased (APP+D vs APP: P=0.041; APP+Gl vs APP: P=0.049; APP + Gm vs APP: P=0.029; APP + Gh vs APP: P=0.036); Among the treatment groups, the GSK-3β

level was significantly higher only in the Gl group than that in the APP+D group (P=0.043). IHC staining pictures were shown in Figure 1(A-F) and detailed data were shown in Table 1.

Table 1 IOD (sum) of GSK-3β in the brain cortex of 7-month-old and 11-month-old mice measured by Immunohistochemistry ($\bar{x} \pm s$)

Group	7-month-old mice IOD (sum)	11-month-old mice IOD (sum)
Control	87625±5257	112080±8966
APP	125228±10515[ab]	131704±11208[ab]
APP+D	102027±4381[ab]	92959±5604[ab]
APP+Gl	113031±6134[abc]	82937±7846[ab]
APP+Gm	98896±8762[ab]	68591±4483[abc]
APP+Gh	107244±1752[ab]	74604±5064[abc]

Notes: control: C57BL/6J mice group given 0.5% carboxymethyl cellulose; APP: APPV717I transgenic mice group given 0.5% carboxymethyl cellulose; APP + D: APPV717I transgenic mice group given donepezil (0.92 mg/kg^{-1} · day^{-1}); APP + Gl: APPV717I transgenic mice group given small dose of GEPT (0.075 g/kg^{-1} · day^{-1}); APP + Gm: APPV717I transgenic mice group given medium dose (0.15 g/kg^{-1} · day^{-1}); APP + Gh: APPV717I transgenic mice group given large dose (0.30 g/kg^{-1} · day^{-1}). IOD: integrated optical density. [a]P<0.05, compared with control group; [b]P<0.05, compared with APP group; [c]P<0.05, compared with APP+D group.

Western blot analysis showed that the density of GSK-3β expression in APP mice increased significantly (compared to control group, P=0.008). However, the GSK-3β expression in donepezil or GEPT treated transgenic mice were all decreased, and there was significant difference between APP + Gh group or APP + D group and the APP group (P=0.05). And there was no significant difference between APP + Gh group and APP+D group. Representative Western blot bands were shown in Figure 2A and detailed data were shown in Figure 2B.

GSK-3β expression levels in11-month-old experimental mice

Immunohistochemistry analysis showed a increase of GSK-3β in brain cortex of 11-month-old APPV717I transgenic mice and C57BL mice (there was no significant difference between APP and control group, P=0.071), while the GSK-3β expression in donepezil or GEPT treated groups were all significantly decreased (APP+D vs APP: P=0.031; APP+Gl vs APP: P=0.023; APP + Gm vs APP: P=0.001; APP + Gh vs APP: P=0.020); Among treatment group, there were significant differences between APP+Gm or APP+Gh group and APP+D group (APP+Gm vs APP+D: P=0.024; APP+Gh vs APP+D: P=0.036), and the APP+Gm or APP+Gh group lower than the APP+D group. IHC staining pictures were shown in Figure 3 (A-F) and detailed data were shown in Table 1.

Western blot analysis also showed that the density of GSK-3β in the treatment groups were all significantly decreased (APP+D vs APP: P=0.021; APP+Gl vs APP: P=0.017; APP + Gm vs APP: P=0.013; APP + Gh vs APP: P=0.026). Representative Western blot pictures were shown in Figure 4A and detailed data were shown in Figure 4B.

DISCUSSION

GSK-3 has gained a great attention in AD pathogenesis research, and it provides a possible link between beta amyloid peptide and tau protein.[4,6] Over activity of GSK-3 can lead to tau hyperphosphorylation and increasing of Aβ was observed in various *in vivo* and *in vitro* models of AD.[5-7] In detail, GSK-3β can phosphorylate certain sites on tau and thereby significantly impair tau's ability to bind microtubules. GSK-3β may also play a role in regulating tau degradation[8] and insoluble tau fibrils formation.[9] And inhibiting GSK-3 activity could relieve accumulation of Aβ peptides and formation of hyperphosphorylated insoluble tau fibrils in the best available models of AD,[10-12] Thus this research aimed to investigate the neuroprotective mechanism of GEPT in treating Alzheimer disease on GSK-3β level.

APPV717I transgenic mice used in this study were of C57BL/6J genetic background and carried mutated human APP-CT100 containing the London mutation V717I, which is characterized by increased generation of Aβ42 and Alzheimer disease-like pathological changes,[18] However, the formation of amyloid plaques only initiates in APP/V717I transgenic mice around their 9 months old.[19,20] In order to investigate the neuroprotecting mechanism of GEPT before and after amyloid plaques formation, APPV717I transgenic mice aged 3 months were used in our experiment and treated with GEPT up to their 7 or 11 months. That is, 3 months old APPV717I transgenic mice were treated by GEPT extract for 4 or 8 months in this study.

In previous studies, GEPT markedly enhanced learning and memory function of AD rat model induced by hippocampal or intravenous injection of Aβ1-42, and significantly improved spatial learning and memory of APPV717I transgenic mice during the 8 months' treatment.[14,15] Further study shown that GEPT extracts can reduce levels of endogenous Aβ peptide in the brain of APPV717I transgenic mice through inhibiting presenilin-1 (PS1) activity rather than beta-secretase 1 (BACE1) and promoting IDE and NEP activity.[14] And previous preliminary clinical study also indicated that a three-month treatment of GEPT had significant effectiveness in improving memory and cognitive impairment and delaying memory decline through one year in 70 patients with aMCI.[21] However, it is unknown for GSK-3β inhibiting effects of GEPT in APP mice.

GSK-3β was detected in the brain cortex of 7 or 11-month-old experimental mice. The level of GSK-3β expression in the brain cortex of APPV717I

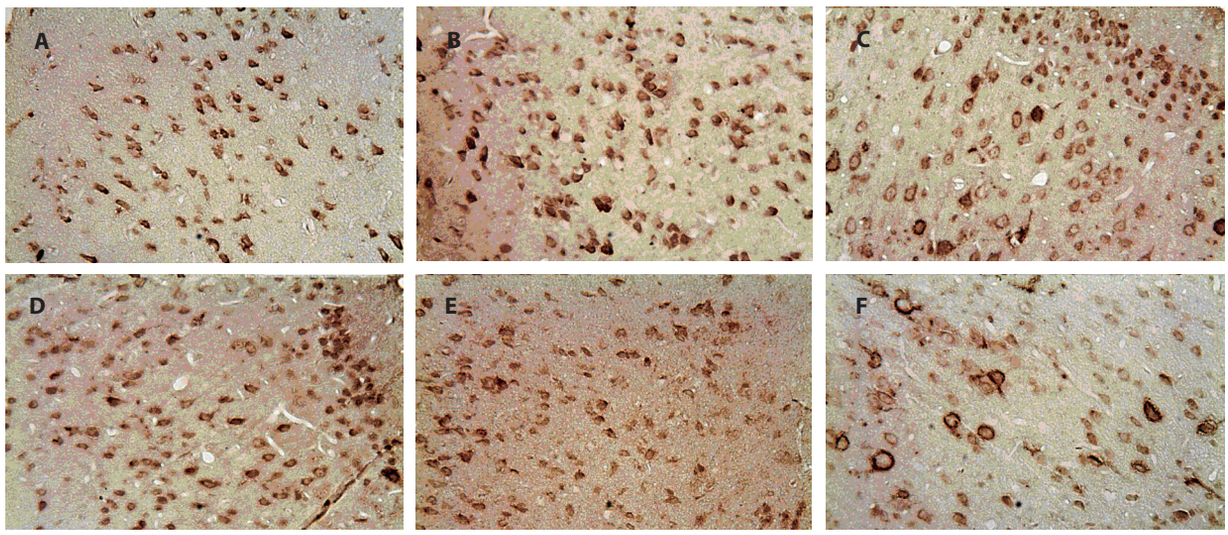

Figure 1 Immunocytochemical staining for GSK-3β in brain cortex of 7-months-old mice (IHC×200, Bar=100 μm)
A: control group; B: APP group; C: APP+D group; D: APP+Gl group; E: APP+Gm group; F: APP+Gh group. Control: C57BL/6J mice given 0.5% carboxymethyl cellulose; APP: APPV717I transgenic mice group 0.5% carboxymethyl cellulose; APP + D: APPV717I transgenic mice group donepezil (0.92 mg/kg^{-1} · day^{-1}); APP+Gl: APPV717I transgenic mice given small dose of GEPT (0.075 g/kg^{-1} · day^{-1}); APP+Gm: APPV717I transgenic mice given medium dose of GEPT (0.15 g/kg^{-1} · day^{-1}); APP+Gh: APPV717I transgenic mice given large dose of GEPT (0.30 g/kg^{-1} · day^{-1}). GSK-3β: glycogen synthase kinase 3β; IHC: immunological histological chemistry staining.

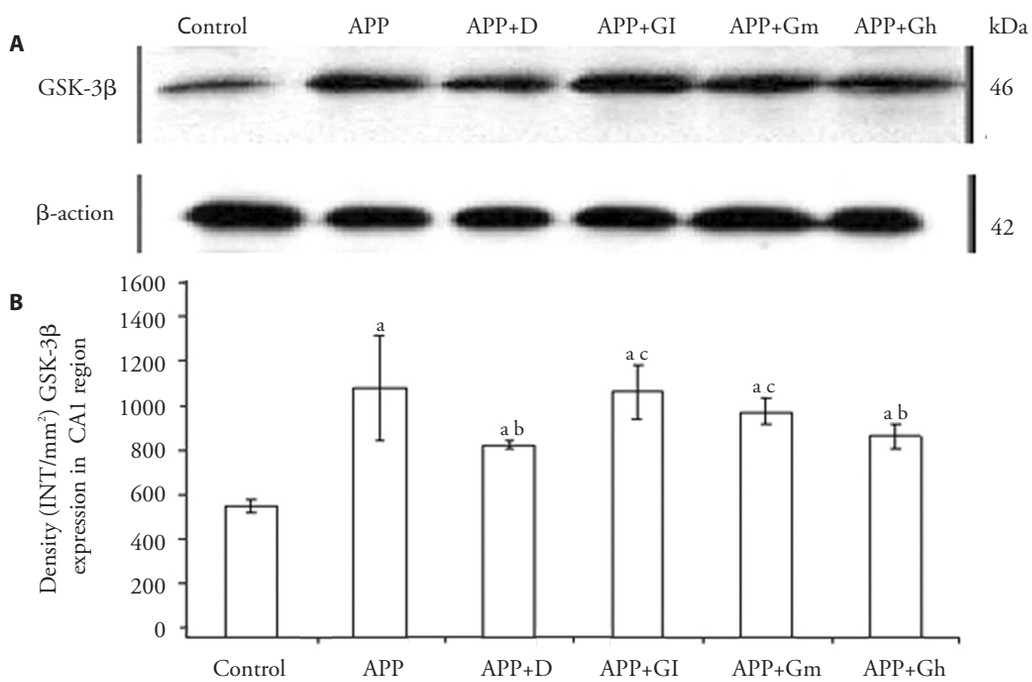

Figure 2 Representative Western blot bands of GSK-3β and β-actin detected by Western blotting in 7-month-old mice (A) and data column analysis (B)
Control: C57BL/6J mice given 0.5% carboxymethyl cellulose; APP: APPV717I transgenic mice group 0.5% carboxymethyl cellulose; APP+D: APPV717I transgenic mice group donepezil (0.92 mg/kg^{-1} · day^{-1}); APP+Gl: APPV717I transgenic mice given small dose of GEPT (0.075 g/kg^{-1} · day^{-1}); APP+Gm: APPV717I transgenic mice given medium dose of GEPT (0.15 g/kg^{-1} · day^{-1}); APP+Gh: APPV717I transgenic mice given large dose of GEPT (0.30 g/kg^{-1} · day^{-1}). GSK-3β: glycogen synthase kinase 3β; INT: intensity. ^{a}P<0.05 compared with control group; ^{b}P<0.05 compared with APP group; ^{a}P<0.05 compared with donepezil group.

transgenic mice was significantly higher than control group, and the GSK-3β expression of donepezil or GEPT treated transgenic mice were all significantly decreased compared with APP groups. Among the treatment groups, there were no significant difference between APP + Gm or APP + Gh group and the APP + D group. This data indicates that GEPT extract can also act as a potential GSK-3β inhibiting agent in APP mice, which can exert before the formation of amyloid plaques.

In summary, GEPT can effectively decrease the GSK-3β expression level in the brain cortex of APPV717I transgenic mice. Moreover, this study showed that the effect of GEPT on GSK-3β in the cerebral cortex of 11 months old mice is more significant than 7 months old mice. It indicates that GEPT could reduce

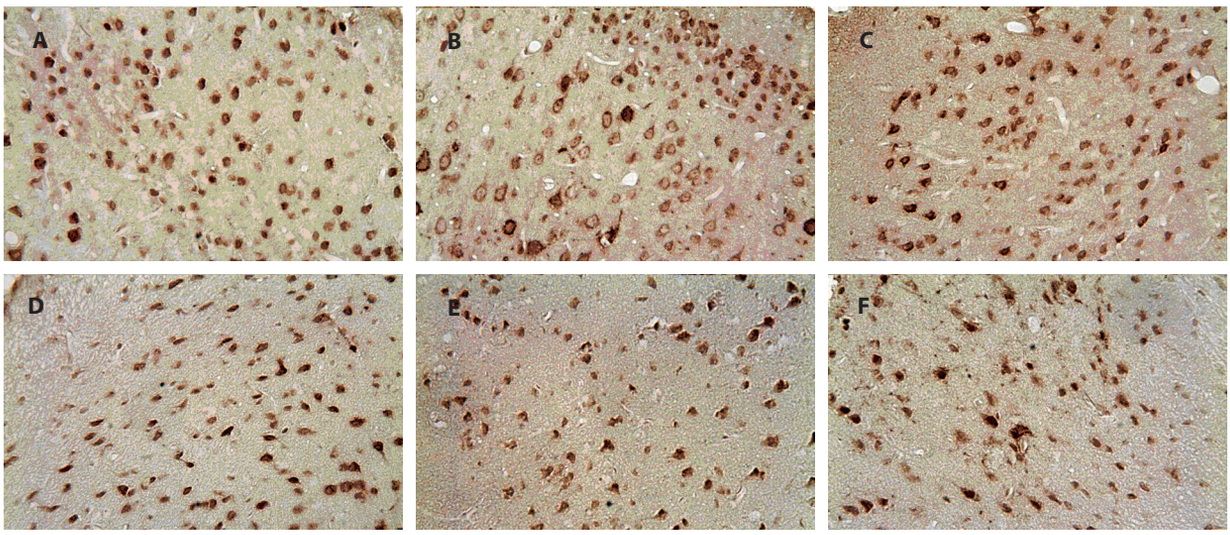

Figure 3 Immunocytochemical staining for GSK-3β in brain cortex of 11-months-old mice (IHC×200, Bar=100 μm)
A: Control group; B: APP group; C: APP+D group; D: APP+Gl group; E: APP+Gm group; F: APP+Gh group. Control: C57BL/6J mice given 0.5% carboxymethyl cellulose; APP: APPV717I transgenic mice group 0.5% carboxymethyl cellulose; APP+D: APPV717I transgenic mice group donepezil (0.92 mg/kg^{-1}·day^{-1}); APP+Gl: APPV717I transgenic mice given small dose of GEPT (0.075 g/kg^{-1}·day^{-1}); APP+Gm: APPV717I transgenic mice given medium dose of GEPT (0.15 g/kg^{-1}·day^{-1}); APP+Gh: APPV717I transgenic mice given large dose of GEPT (0.30 g/ kg^{-1}·day^{-1}). GSK-3β: glycogen synthase kinase 3β; IHC: immunological histological chemistry staining.

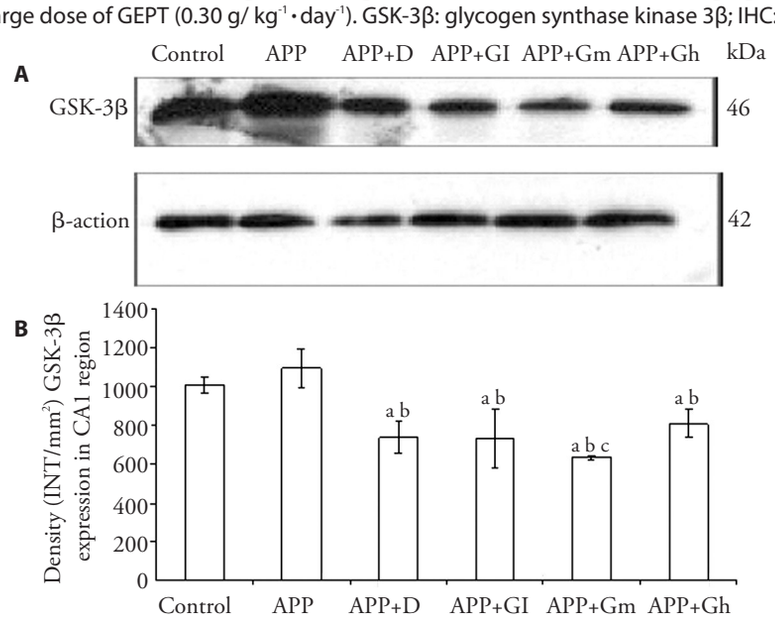

Figure 4 Representative Western blot bands of GSK-3β and β-actin detected by Western blotting in 11-month-old mice (A) and data column analysis (B)
Control: C57BL/6J mice given 0.5% carboxymethyl cellulose; APP: APPV717I transgenic mice group 0.5% carboxymethyl cellulose; APP+D: APPV717I transgenic mice group donepezil (0.92 mg/kg^{-1}·day^{-1}); APP+Gl: APPV717I transgenic mice given small dose of GEPT (0.075 g/ kg^{-1}·day^{-1}); APP+Gm: APPV717I transgenic mice given medium dose of GEPT (0.15 g/ kg^{-1}·day^{-1}); APP+Gh: APPV717I transgenic mice given large dose of GEPT (0.30 g/ kg^{-1}·day^{-1}). GSK-3β: glycogen synthase kinase 3β; INT: intensity. ^{a}P<0.05 compared with control group; ^{b}P<0.05 compared with APP group; ^{c}P<0.05 compared with donepezil group.

the level of GSK-3β in AD model rats. This partially explains the neuroprotective mechanism of GEPT in preventing and treating AD.

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