Regular Article

Yi-Zhi-Fang-Dai Formula Exerts a Protective Effect on the Injury of Tight Junction Scaffold Proteins in Vitro and in Vivo by Mediating Autophagy through Regulation of the RAGE/CaMKKβ/AMPK/mTOR Pathway

Yuanjin Chan, Wenjing Chen, Yanjie Chen, Zhongkuan Lv, Wenbin Wan, Yaming Li,* and Chunyan Zhang* a,b,c

a Geriatrics Department of Chinese Medicine, Huadong Hospital, Fudan University; Shanghai 200040, China; 
b Department of Neurology, Renji Hospital, Shanghai Jiaotong University; Shanghai 200127, China; and c Department of Chinese Medicine, East Hospital, Tongji University; Shanghai 310000, China.

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Alzheimer’s disease (AD) is a chronic neurodegeneration disease that is closely related to the abnormal tight junction scaffold proteins (TJ) proteins of the blood–brain barrier (BBB). Recently, Yi-Zhi-Fang-Dai Formula (YZFDF) had exerted a neuronal protective effect against amyloid peptide (Aβ) toxicity. Still, the therapeutic mechanism of YZFDF in restoring Aβ-induced injury of TJ proteins (ZO-1, Occludin, and Claudin-5) remains unclear. This study aimed to explore the underlying mechanism of YZFDF in alleviating the injury of TJ proteins. We examined the effects of YZFDF on autophagy-related proteins and the histopathology of Aβ in the APP/PS1 double-transgenic male mice. We then performed the free intracellular calcium levels [Ca2+]i analysis and the cognitive behavior test of the AD model. Our results showed that YZFDF ameliorated the injury of TJ proteins by reducing the mRNA transcription and expression of the receptor for advanced glycation end-products (RAGE), the levels of [Ca2+]i, calmodulin-dependent protein kinase β (CaMKKβ), phosphorylated AMP-activated protein kinase (AMPK). Accordingly, YZFDF increased the expression of the phosphorylated mammalian targets of rapamycin (mTOR), leading to inhibition of autophagy (downregulated LC3 and upregulated P62). Moreover, the Aβ1-42 oligomers-induced alterations of autophagy in murine mouse brain capillary (bEnd.3) cells were blocked by RAGE small interfering RNA (siRNA). These results suggest that YZFDF restored TJ proteins’ injury by suppressing autophagy via RAGE signaling. Furthermore, YZFDF reduced the pathological precipitation of Aβ in the hippocampus, and improved cognitive behavior impairment of the AD model suggested that YZFDF might be a potential therapeutic candidate for treating AD through RAGE/CaMKKβ/AMPK/mTOR-regulated autophagy pathway.

Key words Alzheimer’s disease; blood–brain barrier; autophagy; receptor for advanced glycation end product (RAGE); tight junction

INTRODUCTION

Alzheimer’s disease (AD), the leading cause of dementia, is a chronic central nervous system (CNS) neurodegenerative disease. AD, which clinically manifested progressive memory deficit and cognition decline, is recognized by the WHO as a primary issue for public health all over the world. With the growth of numbers in elders, AD is predicted to affect more than 100 million patients worldwide by 2050. Along with the development of clinical drug research in AD still, the critical mechanism of the pathogenesis of AD has not been fully deciphered. As the prevailing “Amyloid Cascade Hypothesis” indicates that amyloid peptide (Aβ) aggregation plays a crucial role in the pathogenesis of AD, Aβ toxicity would induce blood–brain barrier (BBB) dysfunction and thereby reduced cerebral Aβ clearance, leading to cerebral Aβ precipitations. Several clinical studies found that more than 90% of AD patients had the pathological cerebrovascular manifestations, suggesting the BBB dysfunction was closely related to the Aβ precipitations.

BBB is well known that mainly composed of endothelial cells, astrocytes end foot, pericytes, and basement membrane. Thereinto, endothelial cells are characterized by the typical structure of tight junction scaffold proteins (TJ) protein, which are vital structures for protecting the CNS from infections and toxins. In vitro studies had demonstrated that Aβ resulted in apoptosis that happened in brain endothelium cells (BECs) and the injury of TJ proteins, thereby leading to BBB dysfunction. Further investigation of metabolic mechanisms in regulating TJ proteins, such as autophagy, would be needed to shed light on the process of BBB dysfunction on account of Aβ toxicity. Autophagy is an evolutionarily conserved process in which organelles and proteins undergo degradation. When the formation and degradation of autophagic lysosomes are in a dynamic equilibrium, autophagy assures proper maintaining the quality, quantity of proteins in BECs, and vascular homeostasis. However, an excessive autophagic activity induced by Aβ overload has been found to exacerbate the injury of TJ proteins, indicating that autophagy can be detrimental in vascular lesions of AD. With the close relationship between impaired autophagy homeostasis and Aβ toxicity, the modulation of autophagic activity could be a potential target for the restoration of injured TJ proteins in AD. The receptor for advanced glycation end-products (RAGE), a cytomembrane surface receptor that belongs to the immunoglobulin superfamily, plays an essential role in causing Aβ-
induced cell damage, such as inflammation, endoplasmic reticulum (ER) stress, and autophagy in BECs. However, we have not investigated the role of RAGE-mediated autophagy in vascular lesions in detail. Multiple lines of evidence underscore the importance of the calmodulin-dependent protein kinase β (CaMKKβ)/AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) signaling pathway in regulating cellular biology responses such as autophagy. The accumulation of intracellular Ca\(^{2+}\) concentration could activate CaMKKβ/mTOR signaling pathway, leading to inhibition of mTOR and thus induction of autophagy. Of note, two reports provided biochemical evidence suggesting that Aβ42 oligomers could activate AMPK in a CAMKK2-dependent manner in neurons. Furthermore, in vivo, evidence reported that increased cytoplasmic Ca\(^{2+}\) levels occurred as a consequence of RAGE-mediated cell apoptosis and formation of autophagosomes. Herein, we speculated that CaMKKβ/AMPK/mTOR signaling pathway might be the missing links of RAGE-dependent induction of autophagy caused by Aβ.

Considering AD being a multifactorial pathology resulting from a complex network of interrelated and not fully deciphered factors, Traditional Chinese medicine (TCM) is emerged to be a highly anticipated drug candidate due to its unique advantages in multi-targets, multi-component, and multi-pathway effects. Yi-Zhi-Fang-Dai Formula (YZFDF), which prescribed on the underlying theory and pharmacological research of TCM. YZFDF is composed of several compounds, which included bilobalide, ginkgolide A, ginsenoside Rbl, ginsenoside Rg1, cistanoside A, and α-asarone. These main components of YZFDF can alleviate Aβ toxicity and elicit a proper modulation of autophagic activity in the neuron. Relatively, it had demonstrated that ginsenoside Rg1 could ameliorate the endothelial barrier dysfunction and regulated autophagic activity bidirectionally. Moreover, YZFDF had been reported that it could attenuate ERS-mediated neuronal apoptosis by increasing levels of molecular chaperones Hsp70 and Grp78. However, the therapeutic mechanism of YZFDF in restoring the injury of TJ proteins by modulating autophagy remains unclear.

To elucidate the underlying mechanism in cerebrovascular dysfunction, we pre-treated the cultured murine mouse brain capillary cells (bEnd.3) with Aβ\(_{1-42}\) oligomers to mimic AD BBB model in vitro. We first explored the effects of YZFDF on Aβ-induced TJ proteins injury in bEnd.3 cells through inhibiting autophagy via the RAGE-dependent pathway. Afterward, we used the APPsw/PS1dE9 transgenic mice, a transgenic AD model, to verified whether the therapeutic effects of YZFDF were associated with the regulation of RAGE/CaMKKβ/AMPK/mTOR/autophagy pathway. In this study, our data indicated that YZFDF might play a potent role in the therapy and prevention of AD.

MATERIALS AND METHODS

Reagent and Antibodies The primary antibodies, which included Phospho-AMPKα1, AMPK, LC3, RAGE, and β-actin, were purchased from Cell Signaling Technology (MA, U.S.A.). The primary antibodies, which included Zonula occludens-1 (ZO-1), Claudin-5, and Occludin, were purchased from Invitrogen (CA, U.S.A.). The primary antibodies, which included Phospho-mTOR and mTOR primary, and protein markers were purchased from Thermo Scientific (U.S.A.). The primary antibodies, which included CaMKKβ and P62, were purchased from Proteintech (U.S.A.). The Aβ\(_{1-42}\) primary antibodies were purchased from Abcam (Cambridge, U.K.).

Cell Culture and Treatments bEnd.3 murine cerebral endothelioma cell line was cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin, and 100 µM streptomycin (Invitrogen). The bEnd.3 cultures were incubated overnight at 37°C in a humidified 5% CO\(_2\) incubator. Afterward, the subculture was performed within every 2–3d. For all experiments, bEnd.3 cells were seedeed- arrested at a density of 70–80% confluence without a media change. Then, we would replace the culture media with Opti-MEM (Invitrogen) prior to the treatments applied as described below.

Preparation of Aβ\(_{1-42}\) Oligomer Lypophilized human Aβ\(_{1-42}\) (GL Biochem, Shanghai, China) was dissolved in dimethyl sulfoxide (DMSO) (Sigma, U.S.A.) to produce a storage concentration of 2 mM and kept at ~20°C. In order to form Aβ\(_{1-42}\) oligomer, we diluted 2 mM Aβ\(_{1-42}\) in DMSO with Opti-MEM to make up a final concentration of 100 µM and incubated at 4°C for 24h before utilization, as previously described.

Preparation of YZFDF Drug Powder The Four herbs of YZFDF consist of Ginkgo biloba leaves, Ginseng, Cistanches Herba, and grass leaves Sweetflag. We purchased these herbs from Shanghai Hongqiao Pharmaceutical Co., Ltd. (Shanghai, China). The YZFDF decoction liquid was prepared by TCM Preparation Room of Shanghai Geriatric Institute of Chinese Medicine, Shanghai University of Traditional Chinese Medicine. The identification of the leading chemical compounds of YZFDF were performed by the Pharmacy of Shanghai Jiao Tong University School. The procedure of extracting YZFDF described as follows: 500 g of four herbs was subjected twice to extraction with 75% ethanol for two hours. The drugs of the decoction were removed after filtering. Rotary Evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland) concentrated the filtered liquid and used the freeze-drying method to get drug powder of 158.6 g. The YZFDF drug powder was stored at 4°C and dissolved in DMEM at a concentration of 200 mg/mL. The final required concentrations of YZFDF were prepared from the 200 mg/mL solution diluted in DMEM (Gibco, U.S.A.).

Chemical Compounds of YZFDF The herbal extract of YZFDF and the sera of mice sera were detected by using the high-resolution MS-based techniques. The mutual main chemical compounds included bilobalide (PubChem CID: 73581), ginkgolide A (PubChem CID: 9909368), ginsenoside Rg1 (PubChem CID: 135398523), cistanoside A (PubChem CID: 102004878) and α-asarone (PubChem CID: 636822).

Animal Drug Treatments The 30-week-old APP/PS1 (B6C3-Tg (APPswe, PSEN1dE9) Nj2) double-transgenic male mice (purchased from the Nanjing Biomedical Research Institute of Nanjing University, China (SCXK 2015-0001)) were chosen for the AD model. Animals were housed in a 25 ± 1°C with 65 ± 5% humidity laboratory animal room and kept in a 12h reversed light/dark cycle with free fed with a standard diet and water for two weeks due to acclimatization. This study abided the animal welfare and the ethical principles of
animal experimentation and approved by the Animal Ethics Committee of the Department of Laboratory Animal Science of Fudan University.

**Animal Experimental Design**  The mice were randomly assigned into five experimental groups: (1) age-matched control group (transgene-negative wild type (WT) mice treated with saline by gavage, \( n = 10 \)); (2) model group (APP/PS1 mice treated with saline by gavage, \( n = 10 \)); (3) YZFDF-low dosage group (APP/PS1 mice treated with YZFDF extracts at 11.42 \( \mu \)g/kg/d by gavage, \( n = 10 \)); (4) YZFDF-Middle dosage group (APP/PS1 mice treated with YZFDF extracts at 22.84 \( \mu \)g/kg/d by gavage, \( n = 10 \)); (5) YZFDF-High dosage group (APP/PS1 mice treated with YZFDF extracts at 45.68 \( \mu \)g/kg/d by gavage, \( n = 10 \)). The dose was determined based on the dosage conversion coefficient of human and mouse as well as previous studies. The period of YZFDF extracts gavage was 9-month.

**Morris Water Maze (MWM) Test**  After the 9-month YZFDF gavage administration, the MWM test was conducted to test spatial learning and memory ability of all mice (\( n = 10 \)/group). The apparatus was made up of a circular polypropylene pool (120 cm diameter) divided into four equal quadrants. A submerged platform (8 cm diameter) was centered in the third quadrant located 1 ± 0.5 cm below the surface of the white-opaque water (25 ± 0.5°C). In short, the orientation navigation experiment was used to evaluate the learning ability of mice and continued for five consecutive days. Each mouse was allowed to search the platform for 60 s at the same time since the first day. The starting point had been changed for each trial (4 trials per day) daily. When mice failed to locate the platform within the 60 s, mice were guided to the platform and conducted a duration of staying platform for 10 s. The escape latency to the platform of each mouse was calculated as the required time to find the hidden platform. The spatial exploration experiment was conducted in the 60 s for mice on the 6th day by removing the platform. Put each mouse into the pool from the same point. The frequency of animal swimming through the platform location and the duration(s) of target quadrants were recorded and analyzed by SMART 3.0 software.

**Passive Avoidance Test**  The passive avoidance test is a fear-motivated test that is used to appraise the learning and memory ability by passively avoiding electrical shock. It requires the animal to behave in a manner opposite to its innate dark preference. The test was performed by using a shuttle-box apparatus, which consists of a stainless-steel grid floor below the two compartments separated by a door. One compartment was lit with a bright, cold house light and the other compartment was made with a dark, opaque wall and served as the unsafe side. On the first day, the mice were put into the light side for 5 min of acclimatization to eliminate the exploratory reflex. During the process of the test, the mice received an electric shock (0.2–0.8 mA, 2–5 s duration) when they stepped into the dark sides. The performance of the passive avoidance test was tested after 24 h without applying the electrical shock, and the initial latency to enter the dark side was recorded.

**Western Blot Analysis**  Cells were collected at 80–90% confluence and lysed in 90 µL of RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris–HCl (pH 7.4), 20 mM NaF, 20 mM ethylene glycol bis(2-aminoethyl ether)-N,N',N''-tetraacetic acid (EGTA), 1 mM dithiothreitol, 1 mM Na2VO3) with 15 µL of phenylmethylsulfonyl fluoride (PMSF) to yield whole-cell extracts for Western blot analysis. Frozen brain tissues were homogenized in RIPA buffer (60 Hz/s) and were then centrifuged at 12000 × g for 15 min at 4°C after separating the cortex and hippocampus. The supernatants were collected, and total protein levels were measured with the BCA protein assay kit (Beyotime). An equal amount of the protein samples contained (40–50 µg) was denatured and separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was transferred onto a nitrocellulose membrane (NC) during the process of electrophoresis. The proteins were blocked with 5% fat-free milk for 3 h at room temperature and subsequently incubated with primary antibodies overnight at 4°C. After washing three times with TBST, the proteins were incubated with species-specific secondary antibody (Millipore, U.S.A.) for 1 h at room temperature. The images of Western blot bands were visualized using a ChemiDoc XRS+ system fluorescence imaging system (BIO-RAD, U.S.A.).

**Immunohistochemistry**  After six days of the MWM test, five mice in each group were randomly selected for immunohistochemistry. The remaining mice were used for Western blot analysis. Mice were anesthetized with 2% chloral hydrate (0.2 mL/10 g) and transcardially perfused with 50 mL of physiological saline (0.9% NaCl), followed by 50 mL of 4% paraformaldehyde (PFA). After removing the brains from the skull, the brain tissues were performed on 4% PFA-fixed, 4% paraformaldehyde (PFA). After removing the brains from the skull, the brain tissues were performed on 4% PFA-fixed, dehydra tion, transparent, dip wax, embedded, sliced, antigen retrieval brain. The hippocampus sections per mouse (3–5 µm) were prepared and immunostaining with rabbit anti-Aβ primary antibodies (1: 400) overnight at 4°C. A microscope captured images, and quantification was performed in Image-Pro Plus 6.0 and Image J software, and the mean optical density (MOD) of the antibody-immunoreactive area was calculated.

**Quantitative Real-Time PCR (qRT-PCR)**  qRT-PCR assays were performed with the real-time PCR detection system (Eppendorf) according to the manufacturer’s protocol. We isolated the total RNA from cultured bEnd.3 cells and brain tissues by using TRIzol reagent (Invitrogen, CA, U.S.A.) and obtained cDNA with the ReverTra Ace qPCR RT Kit. The amplification process was performed with the SYBR Green RT-PCR Master Mix kit (TOYOBO, Japan). Sequences of the upstream and downstream PCR primers to detect RAGE mRNA used in qRT-PCR were 5'-AAT GAC TCT GCC ACC AGTTC T-3' and 5'-GTT GGG GAC AG A TAA ACT CAA AG-3', respectively. Upstream and downstream primers for GADPH mRNA were 5'-CAG CCA CCC GAG ATT GAG CA-3' and 5'-TAG TAG CGA CCG GCG GTG T-3', respectively.

**Intracellular Calcium Measurements**  Levels of free intracellular calcium ([Ca^2+]i) were measured using the cell-permeable calcium-sensitive fluorescent dye Fluo-3/AM (Sigma, St. Louis, MO, U.S.A.). After pre-treated with YZFDF (20, 50, 100 µg/mL) for 2 h and/or Aβ1-42 (10 µM) for 24 h, cells were collected and incubated with five µM Fluo-3/AM for 60 min at 37°C. After washing the cells with PBS twice, we analyzed the fluorescence intensity of Fluo-3/AM probes with flow cytometry, using BD FACSAria II (Becton Dickinson, U.S.A.) with excitation at 488 nm and emission at 530 nm. Quantification was performed in Flowjo 10 software, and the mean fluorescence intensity was calculated.
Statistical Analysis  All results are expressed as the mean± standard deviation (S.D.). Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, U.S.A.). All experiments were repeated three times independently. Statistical significance of difference among different groups was analyzed by one-way ANOVA or Student’s t-test. A value of $p<0.05$ was considered statistically significant.
RESULTS

Inhibition of RAGE Prevented the Aβ_{1-42} Oligomers-Induced Alterations of Autophagy in bEnd.3 Cells

via Ca^{2+}/CaMKKβ/AMPK/mTOR Pathway

Our previous study indicated that Aβ_{1-42} oligomers-induced disruptions of autophagy via RAGE-dependent pathway still, the underlying mechanism has not been investigated in detail. Given
that overexpression of RAGE would amplify the intracellular concentration of Ca$^{2+}$, we aimed to address the role of Ca$^{2+}$/CaMKK$\beta$/AMPK/mTOR signaling in RAGE-mediated disruption of autophagy. Firstly, we pre-treated bEnd.3 cells with RAGE small interfering RNA (siRNA), followed by A$\beta_{1-42}$ oligomers treatment. As shown in (Fig. 1C), RAGE siRNA downregulated the ratio of LC3-II/LC3-I and upregulated the level of P62 and phosphorylation level of mTOR as compared with A$\beta_{1-42}$ oligomers-treated alone group (Fig. 1A). Therein-to, we further noticed that RAGE siRNA abrogated the elevation of [Ca$^{2+}$]$_i$ (Fig. 1E), CaMKK$\beta$ and phosphorylation level of AMPK induced by A$\beta_{1-42}$ oligomers (Fig. 1A), indicating RAGE-mediated activation of autophagy via Ca$^{2+}$/CaMKK$\beta$/AMPK/mTOR signaling in bEnd.3 cells.

**YZFDF Can Prevent A$\beta_{1-42}$ Oligomers-Induced Impaired Autophagy and TJ Proteins Injury in bEnd.3 Cells**

To investigate the protective effects of YZFDF on A$\beta_{1-42}$ oligomers-induced impaired autophagy and TJ proteins injury, bEnd.3 cells were pre-treated with or without YZFDF for 2 h and then incubated with 10$\mu$M A$\beta_{1-42}$ oligomers for 24 h. As shown in (Fig. 2C), we showed that cells pre-treated with various concentrations of YZFDF downregulated the ratio of LC3-II/LC3-I and upregulated the level of P62 in a dose-dependent manner while A$\beta_{1-42}$ oligomers-treated alone group showed an increased ratio of LC3-II/LC3-I and decreased the level of P62. Simultaneously, YZFDF restored the decreased levels of ZO-1, Occludin, and Claudin-5 induced by A$\beta_{1-42}$ oligomers in a dose-dependent manner (Fig. 2A), indicating YZFDF might prevent injury of TJ proteins by reversing the overexpression of autophagy.

**RAGE/CaMKK$\beta$/AMPK/mTOR Signaling Was Involved in the YZFDF Induced Suppression of Autophagy in bEnd.3 Cells**

Finally, we further explored the potential mechanism by which YZFDF suppressed autophagy. Our previous study demonstrated that RAGE-mediated the A$\beta_{1-42}$ oligomers-induced alteration of autophagy and TJ proteins in bEnd.3 cells through Ca$^{2+}$/CaMKK$\beta$/AMPK/mTOR pathway. However, it is uncertain whether YZFDF alleviates TJ protein damage is related to RAGE-mediated autophagy activation. As indicated in (Figs. 3A, B), YZFDF significantly abrogated the A$\beta_{1-42}$ oligomers-induced increased protein level and mRNA transcription of RAGE in a dose-dependent manner. In contrast, YZFDF increased the phosphorylation level of mTOR as compared with A$\beta_{1-42}$ oligomers-treated alone group, suggesting that YZFDF suppressed autophagic activity through RAGE-dependent pathway. Of note, the elevation of [Ca$^{2+}$]$_i$, CaMKK$\beta$ and phosphorylation level of AMPK induced by A$\beta_{1-42}$ oligomers were significantly reversed by YZFDF as shown in (Fig. 3D), indicating the involvement of RAGE/CaMKK$\beta$/AMPK/mTOR signaling in YZFDF induced regulation of autophagy.

**YZFDF Prevented Cognitive Behavior Impairment of AD Transgenic Mice**

According to the previous study, APPsw/PS1dE9 transgenic mice (Tg) develop amyloid pathol-
ogy at 6-month-old (i.e., the early stage of AD) were selected to assess the effects of YZFDF treatment. After 9 months of treatment of YZFDF, behavior tests were performed on the mice, including Morris water maze and passive avoidance. During the acquisition test, the Tg + NS group exhibited prolonged escape latencies after six days of training compared to the WT + NS group (Figs. 4B, C), but the high concentration of YZFDF treatment prevented the prolonged escape latency in APPswe/PS1dE9 transgenic mice (Figs. 4B, 4C). In the probe test, the percentage of time spent in the target quadrant (Fig. 4E) decreased in the Tg + NS group compared to the WT + NS group, simultaneously YZFDF treatment sig-
Fig. 5. YZFDF Treatment Alleviated Aβ Precipitations in the Hippocampus by Ameliorating Impaired Autophagy and TJ Proteins Injury in AD Transgenic Mice Brains

(A) The levels of Aβ1–42, TJ proteins (ZO-1, Occludin, and Claudin-5) and autophagy marker (LC3 and P62) were determined by Western blot analysis of the cerebral cortex of WT + NS and Tg + NS groups. (B) The levels of RAGE, CaMKKβ, phosphorylated AMPK, and phosphorylated mTOR were determined by Western blot analysis of the cortex of WT + NS and Tg + NS groups. (C, D) Quantitative analysis is normalized against the levels of β-actin (n = 8/group). (E–G) The differentially expressed levels of the TJ proteins (ZO-1, Occludin, and Claudin-5) and autophagy marker (LC3 and P62) were determined by Western blot analysis of the cerebral cortex of YZFDF treated Tg groups. (F–H) Quantitative analysis is normalized against the levels of β-actin (n = 8/group). The dosage of YZFDF in Low conc. group, Middle conc. group, High conc. group were 11.42, 22.84, 45.68 g/kg/d, respectively. Results were shown as the mean ± S.E.M. * YZFDF groups versus Tg + NS: p < 0.05, ** YZFDF groups versus Tg + NS: p < 0.01, *** YZFDF groups versus Tg + NS: p < 0.001, "WT + NS versus Aβ group: p < 0.05, ""WT + NS versus Aβ group: p < 0.001, ""WT + NS versus Aβ group: p < 0.0001.
significantly prevented this decline in a dose-dependent manner (Fig. 4E), suggesting that YZFDF treatment could ameliorate the spatial memory acquisition and retrieval of the APPswe/PS1dE9 transgenic mice. In the passive avoidance test, the Tg + NS group significantly reduced step-through latencies (Fig. 4F) compared to the WT + NS group. While the YZFDF treatment restored these indicators to the average level in a dose-dependent manner, suggesting that YZFDF also improved the fear memory ability of the APPswe/PS1dE9 transgenic mice. Of note, the Aβ precipitation in the hippocampus section of the Tg + YZFDF group decreased in a dose-dependent manner compared with the Tg + NS group was observed at Fig. 4G, indicating that YZFDF prevented cognitive behavior impairment of APPswe/PS1dE9 transgenic mice may closely related to ameliorating Aβ precipitations.

YZFDF Alleviated Aβ Precipitations in the Hippocampus by Ameliorating Impaired Autophagy and TJ Proteins Injury in AD Transgenic Mice Brains

As expected, the levels of ZO-1, Occludin, Claudin-5 in the cerebral cortex of Tg + NS group decreased significantly, but the levels of Aβ1–42 increased significantly compared with the WT + NS group (Fig. 5A), suggesting APPswe/PS1dE9 transgenic mice...
suffered TJ proteins injury induced by Aβ toxicity. Simultaneously, the expression of RAGE, CaMKKβ, phosphorylation of AMPK and the ratio of LC3-II/LC3-I were upregulated in Tg + NS group compared to WT + NS group (Figs. 5A, B), in contrast, the levels of phosphorylation of mTOR and P62 were downregulated in Tg + NS group compared to WT + NS group (Figs. 5A, B), suggesting that RAGE/CaMKKβ/AMPK/mTOR signaling was involved in activation of autophagy in APPswe/PS1dE9 transgenic mice. To determine whether impaired autophagy and TJ proteins injury were reversed by YZFDF in APPswe/PS1dE9 transgenic mice, the ratio of LC3-II/LC3-I and the level of P62, ZO-1, Occludin, Claudin-5 in the cortical areas of brains, which is enriched with brain endothelium cells (BECs), in 15-month-old APPswe/PS1dE9 transgenic mice were determined by Western blot. After 9-month YZFDF treatment, the ratio of LC3-II/LC3-I decreased in a dose-dependent manner in Tg + YZFDF group compared with Tg + NS group was observed at (Fig. 5G), in contrast, the levels of P62, ZO-1, Occludin, Claudin-5 increased in a dose-dependent manner (Figs. 5E, G), suggesting YZFDF might ameliorate injury of TJ proteins by reducing autophagic activity.

YZFDF Ameliorated Impaired Autophagy via RAGE/ CaMKKβ/AMPK/mTOR Signaling in AD Transgenic Mice Brains To determine whether RAGE/CaMKKβ/AMPK/mTOR signaling is involved in YZFDF-induced suppression of autophagy in vivo in this AD model, the levels of RAGE, CaMKKβ and the phosphorylation levels of AMPK, mTOR in the cortical areas of brains in 15-month-old APPswe/PS1dE9 transgenic mice were determined by Western blot. After 9-months YZFDF treatment, the levels of RAGE, CaMKKβ and the phosphorylation level of AMPK decreased in a dose-dependent manner as compared with APPswe/PS1dE9 transgenic controls (Fig. 6A), inversely, the phosphorylation level of mTOR increased in a dose-dependent manner (Fig. 6A), suggesting RAGE/CaMKKβ/AMPK/mTOR signaling might involve in YZFDF induced suppression of autophagy in vivo. Moreover, the mRNA transcription of RAGE in the cerebral cortical area moderately decreased in a dose-dependent manner as compared with APPswe/PS1dE9 transgenic controls (Fig. 6B), which is consistent with the results in YZFDF-treated bEnd.3 cells (Fig. 3B), indicating that YZFDF suppressed RAGE level by decreasing mRNA transcription of RAGE in vivo and in vitro.

DISCUSSION

Our previous study indicated that RAGE-Aβ interaction involved in inducing disruption of TJ proteins in bEnd.3 cells via impaired autophagy,3 but the related signaling pathway had not been elucidated in detailed. In this study, we noticed that RAGE siRNA reversed Aβ induced an increase in intracellular Ca2+ in bEnd.3 cells compared to the Aβ1–42 oligomers-treated alone groups (Fig. 1E), suggesting RAGE played a crucial role in regulating Ca2+-dependent signaling. Much biochemical evidence suggested that RAGE variants were susceptible to degenerative vascular lesions in coronary artery disease (CAD) among the Chinese Han population,33 emphasizing the relationship between RAGE and Ca2+ was closely related to destroying the endothelial barrier. Further evidence suggested that RAGE dysregulated intracellular Ca2+ in neurons upon stimulation of Aβ.12,16 Combined with the findings of our previous study, we speculated that RAGE-induced Ca2+ releasing might regulate the autophagic activity in Aβ-induced injury of TJ proteins. CaMKKβ, which is a well-known Ca2+-dependent kinase and an upstream kinase of AMPK in mammals, abundantly expressed in the brain.32 Numerous studies indicated that an increase in cytoplasmic Ca2+ concentrations stimulated autophagic activity through CaMKKβ/AMPK signaling in SH-SY5Y cells and rat brains.12,14,33,34 AMPK, which is a negative regulator of mTOR signaling, negatively regulates autophagosome formation in the brains of patients.35 As our previous study found that RAGE dysregulated the autophagic activity in bEnd.3 cells through the mTOR-dependent pathway,36 we speculated that CaMKKβ/AMPK/mTOR signaling might involve in RAGE-mediated dysregulation of autophagy. We then measured the levels of CaMKKβ, AMPK, and autophagy-related proteins mTOR, p62, and LC3 by Western blot analysis to test the speculation above. As predicted, RAGE siRNA suppressed the expressions of CaMKKβ and phosphorylation level of AMPK in bEnd.3 cells compared to the Aβ1–42 oligomers-treated alone groups (Fig. 1A), thereby reversing the Aβ-induced downregulation of phosphorylation level of mTOR and level of P62, simultaneously inhibiting the upregulation of LC3-II conversion in bEnd.3 cells (Figs. 1A, C). These data implicated a novel underlying mechanism of cerebral vascular lesions in AD that RAGE-Aβ interaction mediated dysregulation of autophagy in bEnd.3 cells via CaMKKβ/AMPK/mTOR signaling.

Traditional Chinese medicine (TCM) formula has shown unique advantages in the treatment of complex diseases. Further clinical and basic studies had extensively confirmed that TCM formula had the advantages of anti-aging, neuroprotection, regulation of microglial cell function, and clearance of Aβ in AD still.17 The uncertain multi-biochemical component medicines in TCM formula had not been well deciphered. Nowadays, metabolomics is widely used for screening and identifying the functional metabolites of TCM formula.36 Our previous research had identified the mutual bioactive compounds of Ginkgo biloba leaves, including bilobalide and ginkgolide A, and the main compounds of Ginseng, including ginsenoside Rg1 in YZFDF samples and mice plasma by using the high-resolution mass spectrometry (MS)-based techniques.18 Moreover, cistanoside A of Cistanches Herba, and α-asarone of Grass leaves Sweetflag were identified in both YZFDF samples and mice plasma.39 These main components of YZFDF have a wide range of activities, including anti-inflammation,37 promotion of neurogenesis,38 modulation of autophagy,39 which are playing a vital role in the therapy of AD. The previous studies had also revealed that YZFDF had an ameliorative effect against Aβ-induced neurotoxicity through attenuating perturbed protein folding process and ER-stress in SH-SYSY cells,38 suggesting YZFDF might be a potent drug candidate for alleviating the neuronal damage of AD.

In this study, we demonstrated the therapeutic effects of YZFDF on bEnd.3 cells and the AD transgenic mouse model. As predicted, YZFDF markedly prevented Aβ-induced an increase in the ratio of LC3-II/LC3-I and the decreased expression of P62 as shown in (Fig. 2C). On the other hand, the Western blotting results showed that YZFDF restored the downregulated expression of TJ proteins, including ZO-1,
Occludin, and Claudin-5 induced by Aβ in a dose-dependent manner (Fig. 2A), indicating that YZFDF ameliorated Aβ-induced injury of TJ proteins by regulating the impaired autophagy. To test whether the RAGE/CaMKKβ/AMPK/mTOR signaling involved in YZFDF-mediated suppressing autophagy, RAGE, CaMKKβ, AMPK, and mTOR were detected by Western blot analysis and the cytoplasmic Ca2+ was measured by flow cytometric (FCM) analysis. The data showed that elevated expression of RAGE, CaMKKβ, the concentration of Ca2+ and phosphorylation level of AMPK in the Aβ treated alone group was significantly reversed in a dose-dependent manner in YZFDF pre-treated groups (Figs. 3A, D). Simultaneously, the lower phosphorylation level of mTOR was also restored in a dose-dependent manner in YZFDF pretreated groups (Fig. 3A), implicating the RAGE/CaMKKβ/AMPK/mTOR signaling might be a vital pathway in YZFDF ameliorating impaired autophagy and injury of TJ proteins in bEnd.3 cells. Moreover, we measured the mRNA transcription level of RAGE in bEnd.3 cells to explore the regulatory mechanism of YZFDF on RAGE. As predicted, the qRT-PCR result showed that YZFDF decreased the mRNA transcription level of RAGE in a dose-dependent manner (Fig. 3B), indicating YZFDF inhibited RAGE-mediated signaling by suppressing the mRNA transcription level.

Moreover, YZFDF prevented behavioral impairments in the water maze and passive avoidance tests after 9-month treatment (Figs. 4A–F). The results demonstrated that YZFDF treatment effectively improved learning and memory abilities in the APPswe/PS1dE9 transgenic mice. Moreover, YZFDF treatment significantly alleviated Aβ precipitation in the transgenic hippocampus (Fig. 4G) indicated that the YZFDF prevented behavioral impairments of the AD model by attenuating cerebral Aβ precipitation. It is well known that a proper clearance of cerebral Aβ is closely related to the healthy BBB function. Hence, we further examined the levels of TJ proteins in the cerebral cortex, including ZO-1, Occludin, and Claudin-5 by Western blot analysis. As predicted, YZFDF treatment significantly restored the expression of ZO-1, Occludin, and Claudin-5 in a dose-dependent manner compared to the Tg+ NS group (Fig. 5E). However, the underlying protective effect of YZFDF against on injury of TJ proteins in APPswe/PS1dE9 transgenic mice remained unclear. In the present study, we further demonstrated the cerebral autophagic activity and expression of RAGE, CaMKKβ, AMPK, and mTOR in APPswe/PS1dE9 transgenic mice. Consistent with the results of YZFDF pre-treated bEnd.3 cells, YZFDF treatment could inhibit the ratio of LC3-II/LC3-I and levels of RAGE, CaMKKβ, AMPK in the cerebral cortex in a dose-dependent manner compared to Tg+ NS group (Figs. 5G, 6A). On the other hand, the suppressed phosphorylation level of mTOR and level of P62 was significantly reversed in Tg+ YZFDF group compared to Tg+ NS group (Figs. 5G, 6A), suggesting that YZFDF suppressed the autophagic activity in order to prevent the injury of TJ proteins in cerebral cortex via RAGE/CaMKKβ/AMPK/mTOR signaling pathway. Besides, we found that Tg+ high concentration YZFDF group significantly suppressed mRNA transcription level of RAGE compare with the Tg+ NS, which is a similar result in bEnd.3 cells, indicating regulatory mechanism of YZFDF on RAGE is closely related to inhibiting mRNA transcription level of RAGE in vivo and in vitro.

CONCLUSION

It has been reported that dysregulation of TJ proteins would impair the barrier function of BBB, thereby forming a vicious cycle that leading to clearance dysfunction of cerebral Aβ, further aggravating the pathological process of AD. Our previous studies indicated that RAGE-Aβ interaction mediated the disruption of TJ proteins in bEnd.3 cells via impaired autophagy, giving us a clue that modulation of autophagic activity could be a potential target for the restoration of injured TJ proteins induced by Aβ. Our experienced prescription, YZFDF, has shown a proper modulation of autophagy in order to prevent the injury of TJ proteins in vivo and in vitro. Moreover, the data in the present study demonstrated that YZFDF could ameliorate the cognitive behavior impairments and pathological Aβ precipitation in APPswe/PS1dE9 transgenic mice, and these effects might be achieved through YZFDF modulation on autophagy via RAGE/CaMKKβ/AMPK/mTOR signaling pathway. This study showed that YZFDF might be a potent drug candidate for therapy and prevention of AD. However, our present study still could not provide a comprehensive review of the protective effect of YZFDF on the modulation of cerebral Ca2+ flux-dependent autophagy in vivo. Further studies will be performed for functional validation of significantly altered cerebral Ca2+ flux and detection of the liver, renal toxicity of APPswe/PS1dE9 transgenic mice to excavate the detailed therapeutic mechanism and safety of YZFDF on AD intervention.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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