Effect of Yifei Xuanfei Jiangzhuo Formula on the Autophagy Regulatory Mechanism of the AMPK/mTOR/ ULK1 Signaling Pathway in Vascular Dementia Rats (Postprint)

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Date: 2025-09-15T16:39:45+00:00

Abstract

Background: Vascular dementia (VD) is one of the refractory neurological diseases. Currently, the pathogenesis of VD remains unclear, making the identification of key therapeutic targets for VD crucial. Objective: To investigate the intervention effect of Yifei Xuanfei Jiangzhuo Formula (YFXFJZF) on VD rats via the AMPK/mTOR/ULK1 pathway. Methods: Seventy 8-week-old SPF-grade male SD rats were used, with 10 serving as the normal group (K group). The remaining rats were used to establish a VD rat model through staged bilateral common carotid artery ligation. After modeling, rats were randomly divided into VD model group (M group), VD model + YFXFJZF group (Z group), VD model + autophagy inhibitor group (Y group), VD model + autophagy agonist group (D group), VD model + autophagy inhibitor + YFXFJZF group (YZ group), and VD model + autophagy agonist + YFXFJZF group (DZ group), with 10 rats in each group. The Z group received 1.2 g/kg YFXFJZF by gavage, while the K and M groups received equal volume of normal saline by gavage. Starting from day 15 of the experiment, the Y group received intraperitoneal injection of 3-methyladenine 1.5 mg/kg once every other day for 14 days. The D group received rapamycin 2 g/kg by intraperitoneal injection once every other day for 14 days. The total intervention period was 28 days. After behavioral testing, rat blood samples and hippocampal tissues were collected. Hematoxylin-eosin (HE) staining was used to observe pathomorphological changes in hippocampal tissue, and lead citrate staining (electron microscopy) was used to observe hippocampal neurons and autophagosomes. Western blotting was employed to examine the protein expression of AMPK, phosphorylated AMPK (p-AMPK), mTOR, phosphorylated mTOR (p-mTOR), ULK1, phosphorylated ULK1 (Ser757) (p-S757-ULK1), microtubule-associated protein 1 light chain 3

(LC3), and P62 in hippocampal tissue. Real-time quantitative PCR was used to detect the mRNA expression of AMPK, mTOR, ULK1, LC3, and P62 in hippocampal tissue. Measurement data conforming to normal distribution were expressed as (x±s). Inter-group comparisons of homoscedastic data were performed using one-way ANOVA, and pairwise comparisons between groups were conducted using LSD test. Results: Behavioral test results showed that compared with the K group, the escape latency of VD rats in the M group was significantly prolonged, and the platform crossing frequency was significantly reduced ($P<0.05$). Compared with the M group, the escape latency of the Z, Y, YZ, and DZ groups was significantly shortened ($P<0.05$); compared with the M group, the escape latency of the D group was shortened ($P<0.05$), and the platform crossing frequency was significantly increased ($P<0.05$). HE staining results showed that in the K group, cells in the hippocampal CA1 region were arranged neatly with normal morphology and structure. The M group showed severe damage, with deepened cell staining, unclear boundaries between nucleus and cytoplasm, cytoplasmic vacuolation, and irregular morphology. Compared with the M group, the damaged hippocampal tissue was partially repaired in the D, DZ, and YZ groups; while the damaged hippocampal tissue showed more obvious recovery in the Z and Y groups. Lead citrate staining results showed that in the K group, the neuronal cell membranes in the hippocampal CA1 region were intact and smooth, nucleoli were clearly visible, chromatin was evenly distributed within the nucleus, and the nuclear membrane maintained intact morphology. Compared with the K group, the M group showed overall neuronal cell damage, intact cell membranes, cytoplasmic edema, sparsity and dissolution, and obvious organelle swelling; most mitochondria showed significant swelling with intact membranes, massive matrix dissolution, and broken and disappeared cristae. Compared with the M group, neuronal damage was improved and autophagy levels were reduced in the Z, Y, D, YZ, and DZ groups. Western blotting results showed that p-AMPK/AMPK in the M, Y, and D groups was higher than in the K group, while p-AMPK/AMPK in the Z, YZ, and DZ groups was lower than in the M group; p-mTOR/mTOR in the M, Y, D, and DZ groups was lower than in the K group, while p-mTOR/mTOR in the Z and YZ groups was higher than in the M group; p-S757-ULK1/ULK1 in the M and D groups was higher than in the K group, while p-S757-ULK1/ULK1 in the Z, Y, YZ, and DZ groups was lower than in the M group; LC3 /LC3 in the M and D groups was higher than in the K group, while LC3 /LC3 in the Z, Y, YZ, and DZ groups was lower than in the M group; P62 in the M and D groups was lower than in the K group, while P62 in the Z, Y, YZ, and DZ groups was higher than in the M group ($P<0.05$). Real-time quantitative PCR results showed that the mRNA expression of AMPK, mTOR, LC3, P62, and ULK1 in the M group differed significantly from the K group ($P<0.05$); the mRNA expression of AMPK, mTOR, LC3, P62, and ULK1 in the Y and D groups showed no significant difference compared with the M group ($P>0.05$); the AMPK mRNA expression levels in the Z, DZ, and YZ groups were lower than in the M group ($P<0.05$); the mTOR mRNA expression levels in the Z, DZ, and YZ groups were lower than in the M group ($P<0.05$); the LC3 mRNA ex-

pression levels in the Z and YZ groups were lower than in the M group (P<0.05); the P62 and ULK1 mRNA expression levels in the Z, DZ, and YZ groups were lower than in the M group (P<0.05). Conclusion: YFXFJZF may protect hippocampal neuronal function and delay the progression of VD by regulating the AMPK/mTOR/ULK1 signaling pathway and modulating autophagy levels in the hippocampal region.

Full Text

Effect of Yifei Xuanfei Jiangzhuo Prescription on Autophagy Regulation Mechanism of AMPK/mTOR/ULK1 Signaling Pathway in Vascular Dementia Rats

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Abstract

Background Vascular dementia (VD) is one of the refractory neurological diseases, and its pathogenesis remains unclear. Therefore, it is crucial to explore key targets for the treatment of VD. **Objective** To investigate the intervention effect of YFXFJZF (Yifei Xuanfei Jiangzhuo prescription) on vascular dementia (VD) rats based on the AMPK/mTOR/ULK1 pathway. **Methods** A total of 70 8-week-old SPF-grade male SD rats were used, with 10 assigned to the normal group (K group). The remaining rats were used to establish a VD rat model via bilateral common carotid artery ligation. After successful modeling, the rats were randomly divided into the VD model group (M group), VD model group+YFXFJZF group (Z group), VD model group+autophagy inhibitor group (Y group), VD model group+autophagy activator group (D group), VD model group+autophagy inhibitor group+YFXFJZF group (YZ group), and VD model group+autophagy activator group+YFXFJZF group (DZ group), with 10 rats in each group. The Z group was administered YFXFJZF at a dose of 1.2 g/kg via gavage. The K and M groups were given an equal volume of normal saline via gavage. Starting from day 15 of the experiment, the Y group was given intraperitoneal injections of 3-methyladenine (3-MA) at 1.5 mg/kg every other day for 14 days, and the D group was given intraperitoneal injections of rapamycin at 2 g/kg every other day for 14 days. The intervention lasted for 28 days in total. After behavioral testing, blood samples and hippocampal tissues were collected from the rats. Hematoxylin-eosin (HE)

staining was used to observe the pathological changes in the hippocampal tissue. Lead citrate staining (electron microscopy) was used to observe the condition of hippocampal neurons and autophagosomes. Western blotting was used to detect the protein expression of AMPK, p-AMPK, mTOR, p-mTOR, ULK1, p-S757-ULK1, LC3, and P62 in the hippocampus. Real-time quantitative PCR was used to detect the mRNA expression of AMPK, mTOR, ULK1, LC3, and P62 in the hippocampus. Data conforming to a normal distribution were expressed as (x-±s). For comparisons among multiple groups with homogeneous variances, one-way analysis of variance was used, and the LSD method was used for pairwise comparisons between groups. **Results** Behavioral testing showed that compared with the K group, the escape latency of VD rats in the M group was significantly prolonged, and the number of platform crossings was significantly reduced ($P<0.05$). Compared with the M group, the escape latency was significantly shortened in the Z, Y, YZ, and DZ groups ($P<0.05$), and the number of platform crossings was significantly increased in the D group ($P<0.05$). HE staining showed that the cells in the CA1 region of the hippocampus in the K group were neatly arranged with normal morphology and structure. The M group had severe damage, with deeper cell staining, unclear boundaries between nuclei and cytoplasm, cytoplasmic vacuolization, and irregular shapes. The D, DZ, and YZ groups showed some repair of the damaged hippocampal tissue compared with the M group, while the Z and Y groups showed more obvious recovery of the damaged hippocampal tissue. Lead citrate staining showed that the neuronal cell membranes in the CA1 region of the hippocampus in the K group were intact and smooth, with clear nucleoli, evenly distributed chromatin in the nuclei, and intact nuclear membranes. Compared with the K group, the neurons in the M group were severely damaged, with intact cell membranes, edematous cytoplasm, sparse and dissolved cytoplasmic contents, and markedly swollen organelles. Most mitochondria were significantly swollen, with intact membranes, large amounts of dissolved matrix, and broken or disappeared cristae. Compared with the M group, the neuronal damage was improved in the Z, Y, D, YZ, and DZ groups, with reduced autophagy levels. Western blotting results showed that the p-AMPK/AMPK ratio was higher in the M, Y, and D groups than in the K group, while it was lower in the Z, YZ, and DZ groups than in the M group. The p-mTOR/mTOR ratio was lower in the M, Y, D, and DZ groups than in the K group, while it was higher in the Z and YZ groups than in the M group. The p-S757-ULK1/ULK1 ratio was higher in the M and D groups than in the K group, while it was lower in the Z, Y, YZ, and DZ groups than in the M group. The LC3II/LC3I ratio was higher in the M and D groups than in the K group, while it was lower in the Z, Y, YZ, and DZ groups than in the M group. P62 expression was lower in the M and D groups than in the K group, while it was higher in the Z, Y, YZ, and DZ groups than in the M group ($P<0.05$). Real-time quantitative PCR results showed that the mRNA expression of AMPK, mTOR, LC3, P62, and ULK1 in the M group was significantly different from that in the K group ($P<0.05$). There was no significant difference in the mRNA expression of AMPK, mTOR, LC3, P62,

and ULK1 between the Y and D groups and the M group (P>0.05). The mRNA expression of AMPK was lower in the Z, DZ, and YZ groups than in the M group (P<0.05). The mRNA expression level of mTOR was lower in the Z, DZ, and YZ groups than in the M group (P<0.05). The mRNA expression level of LC3 was lower in the Z, YZ, and DZ groups than in the M group (P<0.05). The mRNA expression levels of P62 and ULK1 were lower in the Z, DZ, and YZ groups than in the M group (P<0.05). **Conclusion** YFXFJZF may regulate the level of autophagy in the hippocampus by modulating the AMPK/mTOR/ULK1 signaling pathway, protect the function of hippocampal neurons, and delay the progression of VD.

Keywords Dementia, vascular; Yifei Xuanfei Jiangzhuo prescription; AMP-activated protein kinase; Mechanistic target of rapamycin kinase; UNC-51-like kinase 1; Autophagy

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Introduction

Vascular dementia (VD) is one of the refractory neurological diseases, characterized by decreased computational ability and impaired learning and memory function [1]. The pathogenesis of VD remains unclear, but cellular autophagy caused by chronic ischemia, hypoxia, and hypoperfusion constitutes the pathological basis for memory decline and learning deterioration in VD [2]. Previous studies have shown that Yifei Xuanfei Jiangzhuo Fang (YFXFJZF), guided by the theory of "treating from the lung," has definite clinical efficacy in treating VD, and its neuroprotective effect is related to the improvement of mitochondrial dysfunction and inhibition of inflammatory response [3]. However, molecular biological research on this formula is limited, and its mechanism of action is not yet fully understood. Therefore, this study investigated the neuroprotective mechanism of YFXFJZF in VD rats based on the adenosine monophosphate-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR)/Unc-51-like kinase 1 (ULK1) signaling pathway, to confirm the rationality, effectiveness, and scientific basis of YFXFJZF in preventing and treating VD, thereby providing a scientific basis for its clinical application and promotion, and offering more effective therapeutic targets for VD prevention and treatment.

Methods

1.2 Experimental Drugs and Reagents YFXFJZF (composition: Astragalus 15 g, Ginseng 15 g, Ophiopogon 15 g, Platycodon 10 g, Bitter apricot seed 10 g, Notoginseng powder 15 g, Perilla seed 15 g, Acorus 15 g, Wine-processed rhubarb 6 g) was purchased from the First Affiliated Hospital of Guangxi University of Traditional Chinese Medicine. Electron microscopy fixative (Servicebio, catalog number: G1102), 812 embedding agent (SPI, catalog number: 90529-77-4), hematoxylin staining solution (Zhuhai Besso Biotechnology, catalog number: BA-4041), eosin staining solution (Zhuhai Besso Biotechnology, catalog number:

BA-4024), goat anti-rabbit secondary antibody (ZSGB-BIO, catalog number: ZB2301), primary antibody Anti-AMPK (Wuhan Sanying Biotechnology, catalog number: 10929-2-AP), primary antibody Anti-P-AMPK (Beijing Biosynthesis Biotechnology, catalog number: bs-5551R), primary antibody Anti-MTOR (Wuhan Sanying Biotechnology, catalog number: 28273-1-AP), primary antibody Anti-P-mTOR (Wuhan Sanying Biotechnology, catalog number: 80586-1-RR), primary antibody Anti-ULK1 (Wuhan Sanying Biotechnology, catalog number: 68445-lg), primary antibody Anti-P-ULK1 (Wuhan Sanying Biotechnology, catalog number: 80218-1-RR), reverse transcription kit, and 2×Q3 SYBR qPCR Master mix (Shanghai Tolo Biotech, batch numbers: 24305601, 24429601).

1.3 Main Instruments Transmission electron microscope (Hitachi, model: HT7700), Morris water maze (Shanghai Xinruan, model: ANY-MAZE), grinding mill (Servicebio, model: KZ-II), paraffin microtome (Leica, model: RM2135).

1.4 Experimental Procedures

1.4.1 Animal Grouping and Administration Ten rats served as the normal group (K group). The remaining rats were used to establish a VD rat model using the bilateral common carotid artery ligation method. After modeling, the rats were randomly divided into the VD model group (M group), VD model group + YFXFJZF group (Z group), VD model group + autophagy inhibitor group (Y group), VD model group + autophagy activator group (D group), VD model group + autophagy inhibitor group + YFXFJZF group (YZ group), and VD model group + autophagy activator group + YFXFJZF group (DZ group), with 10 rats in each group. The Z group received YFXFJZF at 1.2 g/kg via gavage, with the dosage calculated according to clinical human-to-rat body surface area conversion [4] for 28 consecutive days. The K and M groups received equal volumes of normal saline via gavage for 28 days. Starting from day 15 of the experiment, the Y group received intraperitoneal injections of 3-methyladenine at 1.5 mg/kg [5] every other day for 14 days, and the D group received intraperitoneal injections of rapamycin at 2 g/kg [6] every other day for 14 days.

1.4.2 Modeling Method Rats were fasted for 8 hours before surgery and anesthetized with intraperitoneal injection of sodium pentobarbital (30 mg/kg). After anesthesia, they were fixed in supine position. The VD model was established by bilateral common carotid artery ligation. Rats in the normal group underwent no surgical procedure.

1.4.3 Behavioral Testing The first 5 days constituted the place navigation test. Rats were randomly placed into the pool from four starting points at different quadrants, and the time required to find the platform within 60 seconds

and remain on it for 5 seconds (i.e., escape latency) was recorded. If a rat failed to reach the platform within 60 seconds, it was manually guided to the platform for 10 seconds, and the escape latency was recorded; each training session lasted 60 seconds. Day 6 was the spatial probe test, during which the platform was removed and rats were placed into the pool from a randomly selected non-platform quadrant. The number of platform crossings within 60 seconds was recorded.

1.4.4 Tissue Collection and Processing (1) Hippocampal tissue collection: After behavioral testing, rats were fasted for 12 hours and anesthetized with intraperitoneal injection of 3% sodium pentobarbital (150 mg/kg). The hippocampus was dissected on ice; one portion was snap-frozen at -80°C, and another portion was fixed in 4% paraformaldehyde for 24 hours for subsequent experiments. **(2) Blood sample collection:** At the end of treatment, rats were fasted for 12 hours before blood and tissue collection. After anesthesia with sodium pentobarbital, abdominal aortic blood was collected. Serum was separated by centrifugation and stored at -80°C until analysis.

1.4.5 Sample Testing (1) Hematoxylin-eosin (HE) staining for pathomorphological observation of hippocampal tissue: Brain tissues were dehydrated, cleared, and paraffin-embedded, then immediately sectioned (3-4 m thickness). After baking, sections were dewaxed with xylene and graded ethanol, stained in hematoxylin solution for 3 minutes, rinsed with water to remove excess stain, transferred to eosin staining solution for 10 seconds, washed with tap water, and finally placed in clean xylene for clearing for 3 minutes. Sections were dried in a 60°C oven for 20 minutes, mounted with neutral resin, pressed flat, baked for another 20 minutes, and observed/photographed under a microscope. **(2) Lead citrate staining (electron microscopy) for observation of hippocampal neurons and autophagosomes:** Hippocampal tissue sections were mounted on egg white-coated slides, air-dried thoroughly, and rinsed with PBS for 5 minutes (repeated 3 times). Sections were fixed with 2.0% osmic acid (60-80 L) for 1.5 hours, rinsed with PBS for 5 minutes (repeated 3 times), dehydrated with graded ethanol (65%, 75%, 85%, 95%, 100%), infiltrated, embedded, sectioned, stained, and observed under transmission electron microscope for hippocampal neurons and autophagosomes. **(3) Western blotting for detection of AMPK, p-AMPK, mTOR, p-mTOR, ULK1, p-S757-ULK1, LC3, and P62 protein expression in hippocampal tissue:** Protein concentration was measured by BCA assay. Hippocampal proteins were denatured and separated by standard SDS-PAGE electrophoresis, then transferred to PVDF membranes. Membranes were blocked with skim milk at room temperature for 1 hour, then incubated with exosome-specific primary antibodies diluted 1:1000 (in 5% skim milk with TBST) at 4°C overnight. After washing with TBST 3 times (10 minutes each), membranes were incubated with secondary antibody at room temperature, washed again with TBST 3 times (10 minutes each), and visualized with Chemi-luminescence substrate

for imaging. The ratios of p-AMPK/AMPK, p-mTOR/mTOR, and p-S757-ULK1/ULK1 were calculated.

1.4.6 Real-time Quantitative PCR Detection of AMPK, mTOR, ULK1, LC3, and P62 mRNA Expression in Hippocampal Tissue Hippocampal tissues from each group were thoroughly ground, TRIzol reagent was added, and total RNA was extracted after homogenization and centrifugation. RNA concentration and purity were measured, followed by reverse transcription and amplification. mRNA expression was calculated using the 2-ΔΔCt method (with GAPDH as internal reference). Reaction conditions were: pre-denaturation at 95°C for 30 seconds, denaturation at 95°C for 10 seconds, annealing/extension at 60°C for 30 seconds, for 40 cycles. Primer sequences were synthesized by Nanning Genesis Biotech (Table 1).

1.5 Statistical Methods Data were analyzed using SPSS 20.0 statistical software. Measurement data conforming to normal distribution were expressed as (x-±s). One-way analysis of variance was used for multi-group comparisons with homogeneous variances, and the LSD method was used for pairwise comparisons between groups. $P<0.05$ was considered statistically significant.

Results

2.1 Morris Water Maze Test Results There were significant differences in escape latency and platform crossing times among groups ($P<0.05$). Pairwise comparisons showed that compared with the K group, the M group had significantly prolonged escape latency and reduced platform crossing times ($P<0.05$). Compared with the M group, escape latency was significantly shortened in the Z, Y, YZ, and DZ groups ($P<0.05$), while the D group showed shortened escape latency and significantly increased platform crossing times ($P<0.05$). There were no significant differences in escape latency and platform crossing times among the Y, YZ, D, and DZ groups ($P>0.05$) (Figure 1 [Figure 1: see original paper], Table 2).

2.2 Effect of YFXFJZF on Hippocampal Pathological Structure in VD Rats In the K group, cells in the hippocampal CA1 region were orderly arranged with normal morphology and structure. The M group showed severe damage with deepened cell staining, unclear nuclear-cytoplasmic boundaries, cytoplasmic vacuolization, no obvious inflammatory cell infiltration, sparse cell arrangement, reduced cell volume, and irregular morphology. Compared with the M group, the D, DZ, and YZ groups showed some repair of damaged hippocampal tissue, while the Z and Y groups showed more obvious recovery with relatively uniform and orderly cell arrangement, though cell morphology and structure remained incomplete, and nuclear-cytoplasmic boundaries were more distinct (Figure 2 [Figure 2: see original paper]).

2.3 Effect of YFXFJZF on Autophagy in VD Rats In the K group, neuronal cell membranes in the hippocampal CA1 region were intact and smooth, nucleoli were clearly visible, chromatin was evenly distributed, and nuclear membranes were intact. The perinuclear space was slightly widened, mitochondria were slightly swollen but intact with orderly cristae, rough endoplasmic reticulum showed mild swelling with minor membrane damage and obvious degranulation, lipofuscin and secondary lysosome structures were visible, and no typical autophagic structures were observed. Compared with the K group, neurons in the M group showed overall damage with intact but continuous cell membranes, moderate cytoplasmic edema, sparse and dissolved cytoplasm, and markedly swollen organelles. Nuclei were elliptical with lightly stained chromatin. Most mitochondria were significantly swollen with intact membranes but extensively dissolved matrix and broken or disappeared cristae. Rough endoplasmic reticulum was slightly dilated with ribosomal detachment, and Golgi apparatus sacculi were slightly dilated. Two autolysosome structures were visible. Compared with the M group, neuronal damage was improved and autophagy levels were reduced in the Z, Y, D, YZ, and DZ groups, with the Z and Y groups showing more pronounced effects (Figure 3 [Figure 3: see original paper]).

2.4 Effect of YFXFJZF on Protein Expression of p-AMPK/AMPK, p-mTOR/mTOR, p-S757-ULK1/ULK1, LC3 II/LC3 I, and P62 in Hippocampal Tissue of VD Rats Significant differences were observed among groups in the expression of p-AMPK/AMPK, p-mTOR/mTOR, p-S757-ULK1/ULK1, LC3 II/LC3 I, and P62 proteins ($P<0.05$). Pairwise comparisons showed that p-AMPK/AMPK was higher in the M, Y, and D groups than in the K group, while it was lower in the Z, YZ, and DZ groups than in the M group. The p-mTOR/mTOR ratio was lower in the M, Y, D, and DZ groups than in the K group, while it was higher in the Z and YZ groups than in the M group. The p-S757-ULK1/ULK1 ratio was higher in the M and D groups than in the K group, while it was lower in the Z, Y, YZ, and DZ groups than in the M group. The LC3 II/LC3 I ratio was higher in the M and D groups than in the K group, while it was lower in the Z, Y, YZ, and DZ groups than in the M group. P62 expression was lower in the M and D groups than in the K group, while it was higher in the Z, Y, YZ, and DZ groups than in the M group ($P<0.05$) (Table 3 , Figure 4 [Figure 4: see original paper]).

2.5 Effect of YFXFJZF on mRNA Expression of AMPK, mTOR, ULK1, LC3, and P62 in Hippocampal Tissue of VD Rats Significant differences were observed among groups in the mRNA levels of AMPK, mTOR, ULK1, LC3, and P62 ($P<0.05$). Pairwise comparisons showed that mRNA expression of AMPK, mTOR, LC3, P62, and ULK1 in the M group differed significantly from the K group ($P<0.05$). There were no significant differences in mRNA expression of these genes between the Y and D groups and the M group ($P>0.05$). The mRNA expression of AMPK was lower in the Z, DZ, and YZ groups than in the M group ($P<0.05$). The mRNA expression level of

mTOR was lower in the Z, DZ, and YZ groups than in the M group (P<0.05). The mRNA expression level of LC3 was lower in the Z, YZ, and DZ groups than in the M group (P<0.05). The mRNA expression levels of P62 and ULK1 were lower in the Z, DZ, and YZ groups than in the M group (P<0.05) (Table 4).

Discussion

The traditional Chinese medicine disease names for vascular dementia include "dai disease" and "forgetfulness," with the brain being the primary affected organ, closely related to the five viscera in TCM theory, and phlegm, stasis, and deficiency being the main pathogenic factors [7-9]. Our research group proposes that VD can be treated by the method of benefiting the lung, dispersing the lung, and descending turbidity, which nourishes the five viscera to achieve the effects of tonifying kidney essence, removing stasis, and resolving phlegm, thereby eliminating pathogenic factors. YFXFJZF can benefit and disperse the lung while also unblocking collaterals and expelling phlegm. Previous clinical and animal experiments [10-11] have confirmed that this formula effectively improves behavioral and memory abilities, with efficacy comparable to the western drug piracetam capsules, and no obvious toxic side effects have been observed. In the formula, Astragalus and Ginseng are used synergistically to tonify qi, calm the spirit, stop fright, open the heart, and benefit intelligence, while also nourishing yin, generating fluids, and lowering qi to moisten the lung. From the perspective of modern pharmacology and pharmacodynamics, the main components of Astragalus—astragalus polysaccharides and astragalus saponins—have been widely applied in anti-VD research [12]. The active components of Ginseng—ginsenosides and ginseng diol—have also been extensively used in VD treatment and research [13]. In addition, Ophiopogon, Notoginseng, Acorus, Rhubarb, Platycodon, Bitter apricot seed, and Perilla seed have been confirmed by numerous studies to have good blood pressure- and lipid-lowering effects [14-15]. Among them, Platycodon disperses lung qi, Perilla seed descends qi and resolves phlegm while moistening the intestines and promoting bowel movements, Notoginseng disperses stasis and unblocks collaterals, Acorus opens orifices with its aromatic dispersing property, and wine-processed rhubarb, bitter and cold, enters the stomach and large intestine meridians, having the effect of purging and descending turbidity. All herbs in the formula exert good intervention and therapeutic effects on VD.

Autophagy is a process of intracellular content degradation and recycling [16]. Studies have found that in VD model rats, severe damage to hippocampal CA1 neurons occurs shortly after ischemia-reperfusion, accompanied by transient elevation of autophagy-related Beclin1 expression. Meanwhile, numerous autophagic vesicles can be detected as early as 8 hours after ischemia, with increased LC3 II expression [17]. To further investigate the role of autophagy in VD, researchers have applied autophagy inhibitors to interfere with the autophagy process. In focal cerebral ischemia model rats, strong autophagic responses were observed in the ischemic penumbra around the infarct area. After

blocking autophagy with the autophagy inhibitor 3-MA, the infarct area was significantly reduced, cerebral edema was alleviated, and escape latency in rats was significantly decreased [18].

AMPK, as a serine/threonine protein kinase, is a key energy sensor that maintains metabolic homeostasis. AMPK and mTOR regulate cerebral ischemia-induced autophagy by coordinately phosphorylating Ulk1. During cerebral ischemia, AMPK is activated, which inhibits mTOR phosphorylation, reduces mTOR binding to the S757-ULK1 site, increases Ulk1-AMPK interaction, activates Ulk1, and induces autophagy [19-20]. LC3 II is formed after autophagy activation, located on both the inner and outer surfaces of the autophagosome membrane, and is specifically expressed as a molecular marker of the autophagosome membrane [21]. The protein expression of LC3 II and the LC3 II/LC3 I ratio are key markers reflecting overall autophagy levels [22]. Studies have shown that LC3 protein expression in the hippocampal CA1 region of VD rats peaks at week 4 [23]. When cognitive function decreases in VD model rats, the expression of related autophagy proteins and autophagy activity in the hippocampus are significantly elevated, indicating that autophagy plays an important role in the occurrence and development of VD [24].

In this study, after YFXFJZF intervention, the degree of pathological damage to rat hippocampal tissue was reduced, the number of autophagosomes and autolysosomes in the hippocampal region decreased, and the levels of p-AMPK/AMPK decreased while p-mTOR/mTOR and p-S757-ULK1/ULK1 levels increased. Combined with the results showing that YFXFJZF could increase LC3 II/LC3 I and P62 protein levels, these findings suggest that YFXFJZF can inhibit AMPK/mTOR/ULK1 pathway activation in VD rats. Furthermore, using the AMPK/mTOR/ULK1 pathway inhibitor 3-MA to inhibit pathway activation on the basis of VD model rats showed that 3-MA alone had no significant effect on inhibiting pro-autophagy in the hippocampal CA1 region of VD rats ($P>0.05$). However, VD rats injected with 3-MA showed significant therapeutic effects after subsequent YFXFJZF intervention ($P<0.05$). Meanwhile, using the autophagy activator rapamycin to activate the AMPK/mTOR/ULK1 autophagy pathway showed that the activator could induce autophagy, with no significant difference in pathway proteins between the D and M groups ($P>0.05$). When YFXFJZF was administered after activator treatment, the results showed that the activator partially counteracted the improvement effects of YFXFJZF on VD, thereby verifying that inhibition of the AMPK/mTOR/ULK1 pathway plays a positive role in YFXFJZF' s protection against hippocampal tissue damage in VD rats.

These results indicate that YFXFJZF exerts anti-autophagy effects by inhibiting the AMPK/mTOR/ULK1 signaling pathway during VD progression, suggesting that YFXFJZF plays an inhibitory role in autophagy. In summary, YFXFJZF can alleviate hippocampal tissue damage and achieve therapeutic effects on VD by inhibiting cellular autophagy, with the AMPK/mTOR/ULK1 pathway being an important target for its autophagy inhibition.

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Author Contributions: Chen Wei: conceptualized the study; Su Mingyang: performed experiments, animal care and testing, and wrote the manuscript; Huang Deqing: collected data; Zhuo Guifeng: collected data; Zhu Xiaomin: performed statistical analysis; Zhang Jinzhi: collected animal tissue samples; Fu Yulan: cleaned experimental equipment; Li Zhongtong: processed figures and wrote the manuscript; Jiang Lingfei: revised the final version and took responsibility for the manuscript.

Conflict of Interest Statement: The authors declare no conflict of interest.

Received Date: 2024-07-12
Revised Date: 2025-06-22

Editor: Zou Lin

Note: Figure translations are in progress. See original paper for figures.