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Complementary Medicine for the Modification of Risk Factors for Cognitive Impairment

Guest Editors: Genevieve Z. Steiner, Sai Wang Seto, Yiu Wa Kwan, Crystal Haskell-Ramsay, and David A. Camfield

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Editorial Board

Contents

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Editorial

Complementary Medicine for the Modification of Risk Factors for Cognitive Impairment

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There is a natural decline in cognitive function as we age, particularly in processing speed and working memory. A range of modifiable factors can increase the risk of accelerated cognitive decline including hypertension, chronic inflammation, atherosclerosis, diabetes, atrial fibrillation, stroke, and impaired central nervous system glucose regulation. Given the lack of adequate interventions for cognitive decline and dementia, it is essential that treatments with the potential to reduce the risk of cognitive impairment are thoroughly explored.

Nutraceutical and lifestyle medicines, including vitamins, herbs, supplements, physical activity, and diet, have been shown to possess anti-inflammatory, antihyperglycaemic, and antihypertensive properties, suggesting their utility in targeting the pathophysiology associated with risk for cognitive decline. The purpose of this special issue was to explore the role of such complementary medicines in the modification of risk factors for cognitive decline.

Authors contributed with a range of papers including original research and review articles spanning in vitro, in vivo, and human studies that improve the understanding of the pathology involved in cognitive impairment in older age and the development of evidence-based complementary treatment strategies for cognitive decline. This collection of works provides a snapshot (that is by no means exhaustive) of current research and some of the emerging trends in this field. This special issue features 11 papers including 4 reviews and 7 original research articles. The complementary therapies explored include nutritional supplements (2 papers), herbal and traditional medicines (6 papers), physical activity (1 paper), and electroacupuncture (2 papers). A brief description of these 11 works is detailed below.

(i) Clinical trials involving nutraceutical and herbal medicine interventions for people with mild cognitive impairment and dementia are reviewed in G. Z. Steiner et al. The manuscript titled "A Systematic Review of Intervention Studies Examining Nutritional and Herbal Therapies for Mild Cognitive Impairment and Dementia Using Neuroimaging Methods: Study Characteristics and Intervention Efficacy" focuses on papers that feature neuroimaging outcome measures.

(ii) D. Chang et al. provide a detailed review of the literature on herbal medicine for vascular dementia in their paper titled "Herbal Medicine for the Treatment of Vascular Dementia: An Overview of Scientific Evidence."

(iii) Vascular disease, such as cerebrovascular disease and atherosclerosis are risk factors for cognitive impairment. X. Zhou et al. review the evidence on this link and promising Traditional Chinese Medicines in their paper titled "Vascular Contributions to Cognitive Impairment and Treatments with Traditional Chinese Medicine."

(iv) The fourth review featured in this special issue summarises the evidence on the efficacy of physical activity in reducing depression. D. C. Mathersul and S. Rosenbaum's paper is titled "The Roles of Exercise and Yoga in Ameliorating Depression as a Risk Factor for Cognitive Decline."

(v) H. Macpherson et al. outline the findings of a clinical trial on older adults in their paper titled "The Effects of Four-Week Multivitamin Supplementation on Mood in Healthy Older Women: A Randomized Controlled Trial."

(vi) The first preclinical paper in this special issue explores the effectiveness of Huannao Yicong extract, a Traditional Chinese Medicine, in reducing tau hyperphosphorylation in Alzheimer's disease model rats. Y. Cao et al.'s paper is titled "Traditional Chinese Medicine Huannao Yicong Decoction Extract Decreases Tau Hyperphosphorylation in the Brain of Alzheimer's Disease Model Rats Induced by $A\beta_{1-42}$."

(vii) X. Wang et al. report their findings from an electroacupuncture treatment on Alzheimer's disease model mice in their paper titled "Improvement of Electroacupuncture on APP/PS1 Transgenic Mice in Spatial Learning and Memory probably due to Expression of $A\beta$ and LRP1 in Hippocampus."

(viii) The second electroacupuncture paper in this special issue explores the effects of this treatment on cerebral hypoperfusion model rats. C.-X. Zheng et al.'s paper is titled "Electroacupuncture Ameliorates Learning and Memory and Improves Synaptic Plasticity via Activation of the PKA/CREB Signaling Pathway in Cerebral Hypoperfusion."

(ix) In the paper titled "Preservation of Cognitive Function by Lepidium meyenii (Maca) Is Associated with Improvement of Mitochondrial Activity and Upregulation of Autophagy-Related Proteins in Middle-Aged Mouse Cortex" by S.-S. Guo et al., maca (a traditional Andean medicine) was found to improve cognition and behavior, and mitochondrial dysfunction in middle-aged mice.

(x) In an in vitro study by L. Liu et al., the effects of $A\beta$-induced neurotoxicity on SH-SY5Y cells are modulated by a the Traditional Chinese Medicine, Yi-Zhi-Fang-Dai formula. The paper is titled "Yi-Zhi-Fang-Dai Formula Protects against $A\beta_{1-42}$ Oligomer Induced Cell Damage via Increasing Hsp70 and Grp78 Expression in SH-SY5Y Cells."

(xi) The final paper in this special issue by M. A. Akhtar et a. titled "Medicinal Plants of the Australian Aboriginal Dharawal People Exhibiting Anti-Inflammatory Activity" explores the medicinal effects of a range of Eucalyptus plants used in traditional Australian Aboriginal medicine by the Dharawal people.

Disclosure

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Acknowledgments

We would like to extend our gratitude to all the authors who submitted their work for consideration in our special issue and to the reviewers for their critical feedback. We hope that this collection of works provides a beacon to help direct the field in the investigation of complementary therapies for the modification of risk factors for cognitive impairment. G. Z. Steiner's contribution to this special issue was supported by funding from an Australian National Health and Medical Research Council (NHMRC)-Australian Research Council (ARC) Dementia Research Development Fellowship (APP1102532). S. W. Seto is supported by a Cardiac Health Institute Research Fellowship.

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Review Article

A Systematic Review of Intervention Studies Examining Nutritional and Herbal Therapies for Mild Cognitive Impairment and Dementia Using Neuroimaging Methods: Study Characteristics and Intervention Efficacy

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Neuroimaging facilitates the assessment of complementary medicines (CMs) by providing a noninvasive insight into their mechanisms of action in the human brain. This is important for identifying the potential treatment options for target disease cohorts with complex pathophysiologies. The aim of this systematic review was to evaluate study characteristics, intervention efficacy, and the structural and functional neuroimaging methods used in research assessing nutritional and herbal medicines for mild cognitive impairment (MCI) and dementia. Six databases were searched for articles reporting on CMs, dementia, and neuroimaging methods. Data were extracted from 21/2,742 eligible full text articles and risk of bias was assessed. Nine studies examined people with Alzheimer's disease, 7 MCI, 4 vascular dementia, and 1 all-cause dementia. Ten studies tested herbal medicines, 8 vitamins and supplements, and 3 nootropics. Ten studies used electroencephalography (EEG), 5 structural magnetic resonance imaging (MRI), 2 functional MRI (fMRI), 3 cerebral blood flow (CBF), 1 single photon emission tomography (SPECT), and 1 positron emission tomography (PET). Four studies had a low risk of bias, with the majority consistently demonstrating inadequate reporting on randomisation, allocation concealment, blinding, and power calculations. A narrative synthesis approach was assumed due to heterogeneity in study methods, interventions, target cohorts, and quality. Eleven key recommendations are suggested to advance future work in this area.

1. Introduction

Dementia is a syndrome comprising over 100 diseases and is characterised by a decline in cognition that interferes with function and independence [1]. Over 46.8 million people worldwide have a diagnosis of dementia [2], and currently there is no cure. Dementia has a heterogeneous pathophysiology, with multiple mechanisms thought to play a role in

the various types. For example, there are several hypotheses on the pathogenesis of Alzheimer's disease (AD) alone (the most common type of dementia, making up approximately 60–80% of all cases [3]) including the amyloid-beta peptide hypothesis, the inflammation hypothesis, the tau hypothesis, and the cholinergic hypothesis [4]. Oxidative stress, hypoxia, calcium imbalance, abnormal metal accumulation, amyloid-beta peptide accumulation within mitochondria, and brain-specific insulin signalling deficiencies are all thought to play a role in the complex pathophysiology of AD [5, 6]. Because of this, first-line single target pharmacological therapies for AD, acetylcholinesterase (AChE) inhibitors (e.g., donepezil) and N-methyl-D-aspartate (NMDA) receptor antagonists (e.g., memantine), are not particularly effective, boosting cognitive function in the early disease stages only, and are unable to slow or stop the disease progression [7, 8].

In the absence of effective pharmaceutical options for dementia, complementary medicines (CMs) have been thoroughly explored. Randomised-controlled trials (RCTs) have been conducted on a range of CMs for dementia, cognitive decline, and mild cognitive impairment (MCI), with many studies currently ongoing. This research has largely focused on nutritional and herbal medicine interventions (e.g., resveratrol, anthocyanins, fish oil, vitamins B and E, *Ginkgo biloba*, *Curcuma longa*, *Bacopa monnieri*, and multi-herb formulas such as Sailuotong [SLT]), dietary interventions (e.g., ketogenic and Mediterranean diets), mind-body interventions (e.g., mindfulness, yoga, tai chi, and other types of physical activity), and manual therapies (e.g., acupuncture), and has yielded mixed results due to a range of methodological inconsistencies. Therapies that show potential as adjunct treatments for dementia, or prevention methods, should be thoroughly investigated with the most rigorous and objective measures to reduce sources of bias.

Neuroimaging techniques can provide an objective, precise, and noninvasive measure of neuronal function and are particularly useful in the assessment of complementary therapies for dementia. Popular functional techniques applied in CM research include electroencephalography (EEG), functional magnetic resonance imaging (fMRI), positron emission tomography (PET), magnetoencephalography (MEG), single photon emission computed tomography (SPECT), and functional near-infrared spectroscopy (fNIRS). Structural magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI) can also be used to assess changes in morphology following longer interventions. As detailed in Table 1, depending on study characteristics such as the sample's degree of cognitive impairment, intervention type and duration, neurocognitive function of interest, and reasons for using neuroimaging, these methodologies have a range of advantages and limitations that should be considered carefully before a specific technique is applied in a CM dementia research study.

Neuroimaging, in particular functional neuroimaging, can be utilised in dementia CM research as a sensitive measure of neurocognition, with the capacity to record changes that cannot otherwise be detected by standard pen-and-paper neuropsychological tests. This is useful given the small effect sizes often reported in CM research, particularly acute studies, and that any proposed intervention for cognitive decline is effectively fighting an uphill battle against neurodegenerative pathophysiology. Furthermore, some techniques can be used to explore the mechanisms of action of a therapy, which is particularly useful in psychopharmacological studies (e.g., nutritional and herbal medicines).

The aim of this systematic review was three-fold: (1) provide a comparison and critical evaluation of the characteristics of studies assessing nutritional and herbal medicines for MCI and dementia; (2) evaluate their use of structural and functional neuroimaging methods; (3) summarise intervention efficacy. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement [9] was followed during the planning, conduct, and writing of this review.

2. Methods

2.1. Eligibility Criteria. Several initial scoping reviews were conducted to determine the eligibility criteria and review scope. Eligibility criteria were determined in line with the PICO principles for systematic reviews [10]:

(i) *Population.* People with cognitive decline, MCI, or dementia

(ii) *Intervention.* Chronic CM treatment

(iii) *Comparisons.* Placebo or control group

(iv) *Outcome.* Structural or functional neuroimaging method

Peer-reviewed studies were included if they reported a herbal or nutritional intervention for MCI or dementia and either structural or functional neuroimaging as an outcome measure. It should be noted that the search strategy was intentionally kept broad and also included both mind-body (e.g., yoga) and manual treatments (e.g., acupuncture); due to the large volume of results, only studies assessing nutritional and herbal interventions were included. Reviews, commentaries, conference proceedings, editorials, preclinical (in vitro and in vivo), and acute clinical studies were excluded, as were studies that were not published in English, or when the full text could not be retrieved.

2.2. Search Strategy. The research team and an experienced librarian reviewed the search strategy before systematic searching commenced. Six databases were searched for studies published in peer-reviewed journals. Abstracts were retrieved from PubMed, ScienceDirect, Web of Science, ProQuest, Scopus, and PsycINFO ranging from databases' dates of inception to August 28, 2016. A full list of keywords and an example of the search strategy for the Scopus database are detailed in Supplementary Material available online at https://doi.org/10.1155/2017/6083629 (Table S1). Similar searches were carried out in the other five databases, with only minor modifications to permit changes in the use of searching symbols. Reference lists of key articles were also searched for other eligible studies.

Table 1: Neuroimaging technique, description, example quantification methods (by no means exhaustive), advantages, limitations, and relevance in CM studies on people with dementia.

Neuroimaging technique	Description	Quantification	Advantages	Limitations	Relevance in CM dementia research
EEG	Quantifies the electrical activity of the brain generated by electrical field potentials from excitatory and inhibitory neuronal activity.	Resting state EEG spectral activity (delta, theta, alpha, beta, gamma): power analyses and scalp-based functional connectivity measures (coherence, phase-lag). Event-related measures: ERPs, EPs, SST, and ERSP including ERS and ERD.	Very high temporal resolution, relatively inexpensive, noninvasive, portable options available.	Poor spatial resolution due to volume conduction.* Not as well-suited to investigations of subcortical dementia.	Captures subtle changes in cognitive and/or sensory function. Allows for the mechanisms of action of CM therapies to be explored. Noninvasive and portable options increase its usability in groups with more significant impairments. Suitable for acute and chronic studies.
fMRI	Measures changes in brain blood flow caused by neuronal activity.	Resting state: Region of interest functional connectivity approach. Event-related: BOLD response.	Good spatial resolution, particularly with high resolution scanners (e.g., 7 T).	Poor temporal resolution: the BOLD response lags by 1-2 s behind the actual neuronal activity. Claustrophobia and high-pitched noises can make scanning uncomfortable for participants.	Captures changes in cognitive and/or sensory function that can have their source localised in the brain.
SPECT	Quantifies changes in brain blood flow and metabolism. Nuclear gamma camera captures a gamma-emitting tracer being absorbed by brain tissue at the same rate as blood flow.	Regional CBF.	Relatively cheap compared to other functional imaging methods (e.g., PET, fMRI).	Administration of radioactive isotope (usually injection) and exposure to gamma radiation. Low spatial resolution (1 cm).	Useful in assessing interventions for dementia as SPECT can differentiate dementia pathologies (e.g., vascular dementia versus Alzheimer's disease). Allows for the mechanisms of action of CM therapies to be explored.
PET	Typically assesses regional brain glucose metabolism by detecting gamma rays emitted by a positron-emitting tracer.	Regional CBF.	Different novel isotopes allow distinction between Alzheimer's pathology and other dementias (PiB-PET).	Administration of radioactive isotope.	Different isotopes allow for tagging of different biochemical processes (e.g., FDG-PET for glucose uptake, or PiB-PET for amyloid imaging). Allows for the mechanisms of action of CM therapies to be explored.
MEG	Measures magnetic fields generated by the electrical activity of the brain.	Similar to EEG, resting state measures (delta, theta, alpha, beta, gamma band power) and event-related measures are available.	Very high temporal resolution, and better spatial resolution (i.e., more accurate) compared to EEG.	Detects only tangential components of current source, so primarily sensitive to activity within sulci.	Captures subtle changes in cognitive and/or sensory function. Allows for the mechanisms of action of CM therapies to be explored. Suitable for acute and chronic studies.

Table 1: Continued.

Neuroimaging technique	Description	Quantification	Advantages	Limitations	Relevance in CM dementia research
fNIRS	fNIRS captures changes in blood flow by detecting haemoglobin concentrations through the transmission and absorption of NIR light.	A range of measures are used, DOT or NIRI being popular forms of fNIR.	Noninvasive and portable.	Limitations when trying to measure activity in subcortical tissue.	CM intervention-associated changes in CBF can be ascertained. Mechanism of action can be explored due to the modulation of haemoglobin.
MRI	Structural MRI images the anatomy of the brain using magnetic fields, radio waves, and field gradients.	Most frequently used measures are voxel-based morphometry and ROI analyses.	Good spatial resolution, particularly with high resolution scanners (e.g., 7 T).	No functional information available.	Volumetric changes in brain regions (or whole brain) can be investigated. Any changes are best explored in chronic studies.
DTI	Measures diffusion of water in order to provide information on tissue microstructures so that white matter pathways within and between brain regions can be explored.	Tractography and tensor estimation.	Exploration of brain networks is becoming increasingly popular within the field. Better resolution with more angles (e.g., 61 direction scan).	No functional information available.	White matter integrity and structural network connectivity can be explored. Any changes are best explored in chronic studies.

Note. BOLD = blood oxygenation level dependent; CBF = cerebral blood flow; CM = complementary therapies; DOT = diffuse optical tomography; DTI = diffusion tensor imaging; EEG = electroencephalograph; EPs = evoked potentials; ERD = event-related de-synchronisation; ERPs = event-related potentials; ERSP = event-related spectral perturbation; ERS = event-related synchronisation; FDG-PET = fluorodeoxyglucose-positron emission tomography; MRI = magnetic resonance imaging; MEG = magnetoencephalograph; NIRI = near-infrared imaging; NIRS = near-infrared spectroscopy; PET = positron emission tomography; PiB-PET = Pittsburgh compound B-positron emission tomography; ROI = region of interest; SPECT = single photon emission computed tomography; SST = steady-state topography.

*Can be addressed to a certain extent in connectivity analyses which partial out instantaneous zero-phase contributions.

Table 2: Risk of bias scale item descriptions.

Risk of bias item	Label	Description
1	Random sequence generation	Was the allocation sequence adequately generated?
2	Allocation concealment	Was allocation adequately concealed?
3	Participant characteristics	Are the characteristics of the participants included in the study clearly described (inclusion/exclusion criteria)?
4	Blinding of participants, personnel, and outcome assessors	Was knowledge of the allocated intervention adequately prevented during the study?
5	Intervention description	Is the intervention of interest sufficiently described to allow replication?
6	Neuroimaging methodology	Are the neuroimaging methods clearly described? Description should include data-acquisition parameters and pre- and postprocessing pipelines.
7	Outcome measurement validity and reliability	Were the outcome measures used accurate and appropriate (valid and reliable)?
8	Selective reporting	Were all outcome measures detailed in the methods reported in the results?
9	Adverse events	Have all important adverse events that may be a consequence of the intervention been reported?
10	Reporting of power calculation and attrition rate effect on power	Was a power calculation reported and was the study adequately powered to detect hypothesised relationships?

Note. Items rated as "yes" were scored as 1. Items rated as "no" or "unable to determine" were both scored as 0. Higher scores indicate a lower risk of bias.

2.3. Data Extraction and Appraisal. One reviewer examined the titles and abstracts of each article. If there was any doubt regarding the eligibility of an article, the full-text was retrieved for clarification. Articles deemed eligible by one reviewer were further assessed by two other independent reviewers to ensure inclusion criteria were met. Any disagreements were resolved by reviewing the full papers and a subsequent discussion.

Study characteristics were extracted from each full-text article. Data extracted included title, authors, publication date, aim, study type, disease focus, study population characteristics, number of participants (target cohort and controls), age (mean/median and SD), gender ratio, participant recruitment, diagnostic criteria, neuroimaging technique and analysis method, neuropsychological test battery, definition and dosage of CM, length of intervention, follow-up, and findings.

An assessment of methodological risk of bias in individual studies was conducted. A 10-item scale was constructed to suit the relevancy of studies in this review. The scale was informed by the Cochrane Handbook [11] and the Quality Checklist for Healthcare Intervention Studies [12] (detailed in Table 2) to capture major sources of bias including selection bias, internal and external validity bias, reporting bias, and statistical bias. For each study, the following elements were assessed: random sequence generation, allocation concealment, sampling, blinding, intervention description, neuroimaging methodology, validity and reliability of outcome measures, selective reporting, adverse events, and statistical power. Each of the 10 items on the scale were rated as yes (scored as 1), no, or unable to determine (both scored as 0), allowing higher scores to indicate a lower risk of bias. Studies with total scores ≥ 9 were considered to have a low risk of bias.

As there was substantial heterogeneity across included studies (in neuroimaging methods, intervention types, and study quality), quantitative analyses (i.e., meta-analysis) were not appropriate. Consequently, this review assumed a qualitative approach with a narrative analysis. The characteristics of each study were extracted, and data were described using a narrative synthesis approach.

3. Results

3.1. Study Selection. Figure 1 illustrates the study selection process. Twenty-one studies [13–33] met the inclusion criteria for review. Three studies [16–18] reported results from the same RCT; the other 18 papers contained unique studies. Ten studies assessed herbal medicines [13, 19, 21, 23, 26, 29–33], 8 focused on vitamins and supplements [15–18, 22, 25, 27, 28], and 3 were on nootropics [14, 20, 23] (i.e., cognitive enhancers).

3.2. Study Characteristics. Table 3 details a summary of the characteristics of the 21 studies including aims, setting, population, intervention type and duration, neuroimaging methods and measures, efficacy of intervention, adverse events, adherence, and retention. Most ($n = 15$) studies included 1 intervention and 1 control group [13–20, 22, 24, 29–33], 1 study had 3 parallel arms [28], and five studies had no control group [21, 23, 25, 26, 34]. Four studies were carried out in China [13, 31–33], 3 each in Japan [23, 26, 30] and the United Kingdom [16–18], 2 each in Italy [27, 28] and Germany [19, 22], and 1 each in the United States [14], Austria [20], Sweden [25], Greece [29], Romania [24], and Korea [21]. One study was a multisite RCT carried out in Belgium, France, Germany, Italy, the Netherlands, and Spain [15]. Three

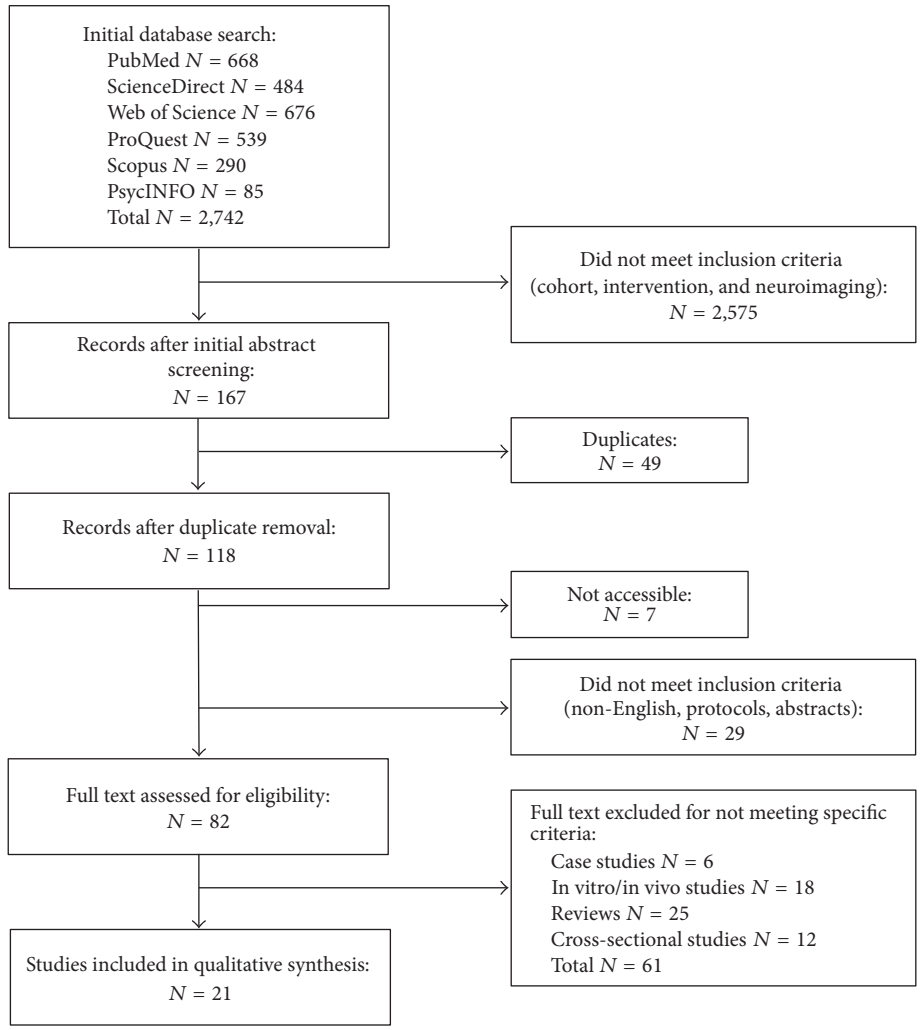

Figure 1: Flow diagram illustrating the study selection process.

studies were published in the 1990s [19, 20, 26], 5 studies were published between 2000 and 2004 [14, 25, 27, 28, 30], and the other 13 were published after 2010 [13, 15–18, 21–24, 29, 31–33].

3.2.1. Participants. Across all the included studies (taking into account the 3 studies on the same RCT [16–18]), the total sample size was $N = 1{,}055$ (476 males, 569 females, 1 study with 10 participants did not specify sex [26]; mean age = 70.9, $SD = 6.8$ years), with individual studies ranging from 8 to 179 participants, and 3 studies with less than 20 participants [21, 23, 26]. Sample size was determined with *a priori* power calculation in 3 studies [15, 18, 20], all of which achieved target sample size.

Nine studies tested 399 participants with AD [13, 15, 19, 21, 23, 25–28], 7 studies examined 319 participants with MCI [16, 17, 22, 23, 29, 31, 32], 4 studies analysed 156 participants with vascular dementia (VaD) [14, 24, 25, 33], 1 study explored 112 participants with unspecified dementia (all cause) [20], 1 study examined 9 participants with mixed-type dementia (combined AD and vascular pathologies) [25], and 1 study included 60 age-matched controls [28]. Twenty studies [13–16, 18–33] measured global cognition at baseline with the mini mental state examination (MMSE: mean score = 22.0, $SD = 2.5$).

3.2.2. Recruitment. Four studies recruited from memory clinics [14, 15, 22, 29], 3 from the community [16–18], 2 from both hospitals and the community [31, 32], 2 from hospital outpatients [25, 30], 2 from hospital inpatients [13, 33], 1 from a medical centre [21], 1 from a nursing home [20], 1 from both outpatient clinics and the community [34], and 1 from a university clinic [23], and 5 did not specify a recruitment location [19, 24, 26–28].

3.2.3. Intervention Design. All studies examined chronic administration with treatment duration ranging from 4 weeks [24, 25] to 2 years [16–18], with most chronic studies ($n = 8$) assessing the effects of a 12-week intervention [13, 19, 21, 26, 30–33]. Ten studies tested herbal interventions [13, 19, 21, 23, 26, 29–33], 8 assessed vitamins (B or E) [16–18, 25, 27, 28] or supplements [15, 22], and 3 tested nootropics [14, 20, 24]. Across all studies, 18 administered an oral intervention,

Table 3: Summary of study characteristics, intervention, neuroimaging, and neuropsychological methodologies, efficacy, and study design.

Author (reference)	Aim, recruitment	Study population	Intervention and duration	Neuroimaging and neuropsychological measures	Efficacy on cognition, neuroimaging measures, any associations, adherence, retention, and adverse events	Study design
Bi et al. (2011) [13], China	Aim: to test the efficacy of fuzhisan (FZS) in people with AD. Recruitment location: hospital patients.	Mild to moderate AD ($N = 25$): randomised to FZS (7M : 5F, 72.50 ± 6.90 yrs, MMSE 19.58 ± 3.23, ADAS-Cog 33.58 ± 6.24, NPI 19.50 ± 6.24) or placebo (6M : 4F, 68.60 ± 6.35 yrs, MMSE 19.70 ± 3.30, ADAS-Cog 34.50 ± 5.80, NPI 19.80 ± 6.11).	Intervention: 1 × 10 g FZS (or placebo) per day, orally. Duration: 12 weeks. Free from over-the-counter medications for at least 2 weeks prior to study entry; free from psychotropic drugs for at least 4 weeks prior.	PET (30 min) NTB: MMSE, ADAS-cog, NPI.	Cognition: significant but small improvements in ADAS-Cog and NPI scores (versus no change in placebo). Neuroimaging: increased or stabilised rCMRglc across frontal, parietal, and temporal cortices, posterior cingulate gyrus, hippocampus, thalamus, cerebellum (versus decreased rCMRglc in placebo). Retention: total 3 withdrew: 1x FZS, 2x placebo (i.e., 88% completion rate). Adverse effects: 2x mild, transient side-effects (1x nausea, 1x constipation).	Randomised, double-blind, placebo-controlled pilot study.
Cohen et al. (2003) [14], USA	Aim: to investigate whether citicoline improves neurocognition & neuroimaging in people with VaD. Recruitment location: specialty care centre (memory & cognitive disorders clinic).	VaD ($N = 30$): randomised to citicoline (7M : 8F, 78.1 ± 5.8 yrs, MMSE 19.0 ± 4.7) or placebo (5M : 10F, 78.0 ± 5.1 yrs, MMSE 21.3 ± 2.9).	Intervention: 2 × 500 mg citicoline (or placebo) per day, orally. Duration: 12 months. Follow-up: 6 & 12 months.	MRI total brain volume MRI SH volume NTB: BNT, COWAT, CVLT, DRS, Grooved Pegboard Test, MMSE, RCFT, TMT, WAIS-R [block design, digit span & symbol, similarities, vocabulary], WMS-R [logical memory, visual reproduction].	Cognition: no difference between citicoline and placebo in neuropsychological performance (i.e., both groups significantly declined from baseline to both 6- and 12-month follow-up). Neuroimaging: no difference between citicoline and placebo in structural brain measures (i.e., both groups had significantly decreased total brain volume & increased SH from baseline to 12-month follow-up). Retention: total 9 withdrew prior to follow-up: 4x citicoline, 5x placebo.	Randomised, double-blind, placebo-controlled trial.
de Waal et al. (2014) [15], Belgium, France, Germany, Italy, The Netherlands, Spain	Aim: to investigate the effect of Souvenaid on brain-activity based networks in people with AD. Recruitment location: AD medical centres.	Mild AD ($N = 179$; drug-naïve): randomised to Souvenaid (45M : 41F, 74.1 ± 6.8 yrs, MMSE 25.1 ± 2.9) or placebo (47M : 46F, 72.5 ± 8.0 yrs, MMSE 25.4 ± 2.7).	Intervention: 1 × 125 mL drink (active Souvenaid or isocaloric control) per day. Duration: 24 weeks. Follow-up: 12 & 24 weeks.	EEG functional connectivity networks NTB: MMSE, COWAT, RAVLT, TMT, WMS-R [digit span, verbal paired associates].	Cognition: no relationship between EEG network and NTB memory performance. Association between beta activity and memory performance at midpoint in the treatment group only. Neuroimaging: decreased beta network EEG in the placebo but not Souvenaid group. *Suggests improved synaptic integrity and function and counteraction of network decline*. Retention: total 12 withdrew prior to follow-up; 14 excluded due to protocol deviations &/or <80% compliance: 4x Souvenaid, 10x placebo.	Randomised, double-blind, placebo-controlled, multisite trial.

Table 3: Continued.

Author (reference)	Aim, recruitment	Study population	Intervention and duration	Neuroimaging and neuropsychological measures	Efficacy on cognition, neuroimaging measures, any associations, adherence, retention, and adverse events	Study design
Douaud et al. (2013) [16], Jernerén et al. (2015) [17] and Smith et al. (2010) [18], UK	Aim: (1) to investigate the effect of B-vitamin treatment on brain atrophy in people with MCI. (2) To investigate the effect of (a) plasma ω-3 fatty acid concentrations, (b) plasma tHCy concentrations, on B-vitamin treatment of brain atrophy in people with MCI. Recruitment location: community.	MCI (N = 156) [15]: randomised to B-vitamin (33M : 47F, 77 ± 5 yrs, MMSE 28.5 ± 1.5) or placebo (27M : 49F, 76 ± 4 yrs, MMSE 28.5 ± 1.5). MCI (N = 168) [19, 20]: randomised to B-vitamin (35M : 50F, 77.0 ± 5.2 yrs, MMSE 28.3 ± 1.8) or placebo (31M : 52F, 76.2 ± 4.5 yrs, MMSE 28.3 ± 1.5).	Intervention: high dose B-vitamin treatment (folic acid 0.8 mg/d, vit. B6 (pyridoxine HCl) 20 mg/d, vit. B12 (cyanocobalamin) 0.5 mg/d). 1x active or placebo tablet per day. Duration: 24 months.	MRI regional grey matter volume [15] MRI whole brain atrophy [19, 20] NTB: CDR-SOB, MMSE, HVLT-R (delayed recall), category fluency (animals).	Neuroimaging: significantly reduced brain atrophy (0.5% versus 3.7%) in posterior brain regions (bilateral hippocampus, parahippocampal gyrus, retrosplenial precuneus, lingual and fusiform gyrus, cerebellum). Rate of brain atrophy was significantly slower (by 29.6%) with B-vitamin treatment than placebo. This effect was even greater (53% lower atrophy) for individuals with high baseline tHcy, a risk factor for brain atrophy. This effect was also even greater (40% lower atrophy) for individuals with high baseline ω-3 fatty acid concentrations, a risk factor for brain atrophy. Adherence: >78% took at least 75% of the tablets; 81.4% (70/84 active, 66/83 placebo) determined biologically compliant via blood samples. Retention: total 20 withdrew (11x B-vit, 9x placebo). Total 15 lost to death or cancer (7x B-vit, 8x placebo). Further 8 lost or excluded for "miscellaneous" reasons (5x B-vit, 3x placebo). Adverse effects: no significant safety issues or group differences in adverse events.	Randomised, double-blind, placebo-controlled trial (VITACOG study).
Heo et al. (2016) [21], Korea	Aim: To investigate the effect of Korean red ginseng (KRG) on brain activity in people with AD. Recruitment location: medical centre.	AD (N = 14; 3M : 11F, 74.93 ± 7.63 yrs, K-MMSE 19.93 ± 4.80).	Intervention: 4.5 g/d KRG, orally (total powder capsule, 6-yr-old root; KT&G Corporation, Daedeok District, Korea; 8.54% Ginsenosides). Duration: 12 weeks.	qEEG (resting, eyes closed) NTB: MMSE, FAB	Cognition: significant improvement in cognitive function (FAB). Neuroimaging: relative alpha power increased in temporal regions for responders versus nonresponders. *Suggests increased frontal lobe function*.	Open, case series study.
Hofferberth (1994) [19], Germany	Aim: to investigate the effect of *Ginkgo biloba* special extract (GBE; EGb 761) treatment on brain activity in people with AD.	AD (N = 42): randomised to GBE (14M : 7F, 63.6 yrs) or placebo (14M : 7F, 63.6 yrs).	Intervention: 80 mg/d GBE (or placebo), orally. Duration: 3 months. Follow-up: 1, 2, and 3 months.	EEG (theta/alpha quotient) NTB: memory, attention, choice reaction time.	Cognition: significant improvement in cognitive function following 1 month and 2 months and maintained following 3 months of GBE. Significant improvement in choice reaction time following 1 month and maintained following 2 and 3 months of GBE. Neuroimaging: significant reduction in theta wave component of theta/alpha quotient following 1 month and maintained following 2 and 3 months of GBE.	Randomised, double-blind, placebo-controlled trial.

Table 3: Continued.

Author (reference)	Aim, recruitment	Study population	Intervention and duration	Neuroimaging and neuropsychological measures	Efficacy on cognition, neuroimaging measures, any associations, adherence, retention, and adverse events	Study design
Köbe et al. (2015) [22], Germany	Aim: to investigate the combined effects of omega-3 fatty acids (FA), aerobic exercise, and cognitive stimulation on brain atrophy in people with MCI. Recruitment location: memory clinics.	MCI ($N = 22$): randomised to target intervention (9M : 4F, 70.0 ± 7.2 yrs, MMSE 28.5 ± 1.1) or control intervention (5M : 4F, 70.0 ± 5.2 yrs, MMSE 27.9 ± 1.7).	Intervention: both target and control groups received omega-3 FA (2.2 g/d; 4x oral capsules daily). Target intervention: aerobic training (cycle ergometer; 2 × 45 min/wk) + cognitive stimulation (AKTIVA: *Aktive Kognitive Stimulation-Vorbeugung im Alter* (active cognitive stimulation-prevention in the elderly); 1x individual + 12x group sessions, 90 min duration, plus daily home practice, beginning week 4). Control intervention: nonaerobic training (stretching & toning; 2 × 45 min/wk). Duration: 6 months.	MRI brain volume NTB: digit span, TMT, Stroop, AVLT, verbal fluency.	Cognition: no change in executive function, memory, sensorimotor speed, or attention. Neuroimaging: increased or reduced atrophy in GM volume for target versus control intervention (middle and superior frontal cortices, frontal pole, angular cortex, posterior cingulate cortex). Significant decrease in total homocysteine concentration. Retention/adherence: original $N = 35$; $n = 13$ discontinued due to time constraints or poor compliance. Adverse effects: None.	Randomised controlled trial
Matsuoka et al. (2012) [23], Japan	Aim: to investigate the effect of toki-shakuyaku-san (TSS) on rCBF in people with MCI or AD. Recruitment location: university clinic.	MCI/AD ($n = 8$; 3M : 5F, 77.8 ± 4.9 yrs, MMSE 23.4 ± 3.6).	Intervention: 7.5 g TSS, orally (powder). Duration: daily for 8 wks.	rCBF (SPECT) NTB: MMSE, NPI, PSMS.	Cognition: no change in MMSE scores (trend toward improved orientation to place). Neuroimaging: significant increase in rCBF in posterior cingulate. Retention/adherence: original $N = 13$; $n = 5$ discontinued due to poor compliance, change in location, or withdrawal. Adverse effects: none.	Open, case series study.
Muresanu et al. (2010) [24], Romania	Aim: to investigate persistence of the effects of cerebrolysin on cognition & qEEG in people with VaD.	VaD ($N = 33$): randomised (2 : 3 : 3) to cerebrolysin 10 mL (4M : 9F, 72.46 ± 2.80 yrs, MMSE 18.92 ± 1.32), cerebrolysin 30 mL (7M : 4F, 70.36 ± 3.62 yrs, MMSE 20.27 ± 1.92), or placebo (5M : 4F, 71.89 ± 3.52 yrs, MMSE 18.89 ± 1.81).	Intervention: 50 mL i.v. infusions of cerebrolysin (10 mL + 40 mL saline or 30 mL + 20 mL saline) or placebo (saline) 5 days/wk. Duration: 4 weeks. Follow-up: 12 weeks (±1).	qEEG, eyes closed resting state NTB: MMSE, ADAS-cog.	Cognition: significant improvement in cognitive performance maintained at follow-up. Neuroimaging: significant (dose-dependent) reduction in qEEG power ratio maintained at follow-up. Retention/adherence: original study $N = 41$; $n = 8$ lost to follow-up or due to poor compliance (i.e., receiving new drug treatment).	Open-label extension of a randomised, double-blind, placebo-controlled trial.

Table 3: Continued.

Author (reference)	Aim, recruitment	Study population	Intervention and duration	Neuroimaging and neuropsychological measures	Efficacy on cognition, neuroimaging measures, any associations, adherence, retention, and adverse events	Study design
Nilsson et al. (2000) [25], Sweden	Aim: to investigate the effect of cobalamin (vitamin B12) treatment on brain function in people with a medical history of cognitive deterioration. Recruitment location: hospital outpatients.	Mild to severe dementia ($N = 29$ (VaD: $n = 13$; AD: $n = 7$, mixed $n = 9$); 15M : 14F, 78.9 ± 6.8 yrs; MMSE 9–23).	Intervention: intramuscular injection of hydroxycobalamin (vit. B12); 1 mg every second day, total 10x. Duration: 1 month.	rCBF (xenon 133 inhalation and cortexplorer with 254 scintillation detectors) NTB: MMSE, OBS.	Cognition: $N = 15$ classified as "clinically improved" (orientation to time and space, recent memory), though dementia severity did not change. $N = 14$ did not show any clinical improvements. Neuroimaging: $N = 15$ "clinically improved" had significant increase in general blood flow level. $N = 14$ slight trend towards a decrease in blood flow.	Open, case series study.
Oishi et al. (1998) [26], Japan	Aim: to investigate the effect of traditional Chinese medicine treatment on brain function in people with AD.	AD ($N = 10$; 65 ± 8 yrs; MMSE 16.0 ± 5.1).	Intervention: traditional Chinese medicine (astragalus root 8 g, *Prunella vulgaris* 3 g, pueraria root 9 g, *Lycii fructus* 8 g, cnidium rhizome 5 g, rhubarb 1 g, alisma rhizome 6 g, peach kernel 6 g, ginseng 3 g, oyster shell 8 g). Duration: 3 months.	ERP (auditory oddball P300) rCBF (stable xenon CT method) NTB: MMSE.	Cognition: significant improvement in MMSE scores (though still below normal range). Neuroimaging: significantly improved (shortened) P300 latency. Increased white matter CBF. Adverse effects: none.	Open, case series study.
Onofrj et al. (2002) [27], Italy	Aim: to test the effects of donepezil (DPZ) versus vitamin E on brain function in people with varying severities of AD.	Mild to severe AD ($N = 60$ completed): first divided into mild AD (group I) versus moderate-severe AD (group II) then randomised to treatment: group I DPZ (6M : 9F, 65.2 ± 1.8 yrs, MMSE 21.5 ± 0.4, ADAS-Cog 22.3 ± 1.0), Group I vit E (7M : 8F, 65.5 ± 1.7 yrs, MMSE 21.5 ± 0.6, ADAS-Cog 22.5 ± 0.9), Group II DPZ (6M : 9F, 66.7 ± 1.5 yrs, MMSE 11.5 ± 0.6, ADAS-Cog 44.5 ± 1.2), Group II vit E (8M : 7F, 66.5 ± 1.6 yrs, MMSE 11.6 ± 0.4, ADAS-Cog 43.5 ± 1.4).	Titration: 14 days, 5 mg/day DPZ or 1000 IU/day vit E, orally. Intervention: 10 mg/day DPZ or 2000 IU/day vit E, orally. Duration: 6 months.	ERP (P300 auditory oddball) NTB: MMSE, ADAS-cog, WAIS.	Cognition: DPZ: significant improvement in neuropsychological test performance, regardless of AD severity, though more pronounced for moderate-severe than mild AD. Vitamin E: severe deterioration of neuropsychological test performance, regardless of AD severity. Neuroimaging: DPZ: significantly reduced P300 latency, regardless of AD severity, though more pronounced for moderate-severe than mild AD. Vitamin E: significantly increased P300 latency, regardless of AD severity. Retention/adherence: total 7 withdrew during initial titration phase (6x DPZ (adverse effects), 1x Vit E (noncompliance)). Adverse effects: 6x DPZ (3x nausea or abdominal discomfort, 3x confused agitation).	Pseudo-randomised, double-blind, controlled trial.

Table 3: Continued.

Author (reference)	Aim, recruitment	Study population	Intervention and duration	Neuroimaging and neuropsychological measures	Efficacy on cognition, neuroimaging measures, any associations, adherence, retention, and adverse events	Study design
Saletu et al. (1995) [20], Austria	Aim: to test the efficacy of nicergoline (NIC) in people with unspecified dementia (all-cause). Recruitment location: nursing home for seniors.	Mild to moderate dementia ($N = 112$, MMSE 13–25; equal distribution SDAT : MID ($n = 56$), equally randomised ($n = 28$) to placebo control (PLAC) or treatment (NIC)): SDAT/NIC: 5M : 23F, 78 ± 7 yrs; SDAT/PLAC: 7M : 21F, 77 ± 10 yrs; MID/NIC: 6M : 22F, 81 ± 7 yrs; MID/PLAC: 9M : 19F, 79 ± 7 yrs.	Intervention: 2 × 30 mg NIC (or placebo) per day, orally. Duration: 8 weeks NIC or placebo, 2-week washout period.	EEG mapping (3 min V-EEG) ERP (P300 auditory oddball) NTB: MMSE, SCAG, CGI.	Cognition: significant improvements in CGI, MMSE, and SCAG (versus pretreatment and placebo group). Neuroimaging: decreased relative power delta/theta and alpha-1, increased relative power alpha-2 and beta (right temporal to frontotemporal and left parietal and temporo-occipital regions) (versus opposite effects in placebo). *Suggests improved vigilance.* Acceleration of total centroid power spectrum (versus pre- and placebo). Shortened latency of P300 (versus pre- and placebo). *Suggests improved information processing.* Retention: total 14 withdrew: 4x SDAT/NIC, 4x SDAT/PLAC, 4x MID/NIC, 2x MID/PLAC. Responder to nonresponder ratio: SDAT/NIC 16 : 8 (i.e., 66.6% responders), SDAT/PLAC 8 : 16, MID/NIC 17 : 7 (i.e., 70.83% responders), MID/PLAC 7 : 19. Adverse effects: 9 of 48 NIC reported adverse effects (mild or marked insomnia (×2), mild itching, blocked nose, sweating, dry mouth, diarrhea, weight loss, constipation, moderate rigor).	Randomised, double-blind, placebo-controlled crossover trial.
Thomas et al. (2001) [28], Italy	Aim: to test the effects of donepezil (DPZ) versus vitamin E versus rivastigmine (Riv) on brain function in people with AD.	Mild to moderately severe AD ($N = 60$): randomised to double-blind treatment (DPZ (9M : 11F, 66.50 ± 9.19 yrs, MMSE 16.0 ± 0.5, ADAS-Cog 33.34 ± 2.70) or vit E (10M : 10F, 65.50 ± 10.61 yrs, MMSE 16.0 ± 0.5, ADAS-Cog 33.45 ± 2.60)), or open trial Riv (9M : 11F, 65.00 ± 8.49 yrs, MMSE 16.0 ± 0.5, ADAS-Cog 33.39 ± 2.70). Age-matched control group ($N = 60$): 25M : 35F, 67.50 ± 14.85 yrs, MMSE 29.0 ± 0.4, ADAS-Cog 14.25 ± 0.50.	Titration: 1 month, 5 mg/d DPZ, 2000 IU/d vit E, or 1.5 mg/d Riv, orally. Intervention: 10 mg/d DPZ or 2000 IU/d vit E, orally. Riv: 1x capsule 2x/d; month 2: total 3 mg/d, month 3: total 6 mg/d, month 4: total 9 mg/d, months 5 & 6: total 12 mg/d. Duration: 6 months. Follow-up: each month.	ERP (P300 auditory oddball) NTB: MMSE, ADAS-Cog, WAIS subscales.	Cognition: DPZ and Riv: significant improvement in neuropsychological test performance. Vitamin E: severe deterioration of neuropsychological test performance. Neuroimaging: DPZ and Riv: significant reduction in P300 latency (no difference between). Vitamin E: significantly increased P300 latency. P300 latency changes were significantly correlated with neuropsychological test scores. Retention/adherence: total 4 withdrew from Riv (3x nausea, 1x noncompliance). Total 2 excluded from vit E (no detectable P300). Adverse effects: 3x nausea.	Randomised three-arm trial with one open-label arm and two double-blind arms.

Table 3: Continued.

Author (reference)	Aim, recruitment	Study population	Intervention and duration	Neuroimaging and neuropsychological measures	Efficacy on cognition, neuroimaging measures, any associations, adherence, retention, and adverse events	Study design
Tsolaki et al. (in press) [29], Greece	Aim: to test the effects of *Crocus sativus* L. (saffron) on brain function in people with aMCI. Recruitment location: outpatient memory & dementia clinic.	aMCI ($N = 35$): *Crocus* (5M : 12F, 71.47 ± 6.73 yrs, MMSE 27.41 ± 1.70, MoCA 22.91 ± 3.00) or wait-list control (4M : 14F, 69.72 ± 7.33 yrs, MMSE 27.89 ± 1.84, MoCA 22.81 ± 2.64).	Intervention: Crocus (no further information available). Follow-up: 12 months.	MRI (global maxima of case "a"; $n = 8$ subgroup) ERP (P300 novelty auditory oddball using HD-EEG (256 channel); $n = 6$ subgroup) NTB: MMSE, MoCA, NPI, activities of daily living.	Cognition: significant improvement in MMSE (versus nonsignificant decline in the wait-list group). Neuroimaging: significantly greater left temporal inferior gyrus volume. Significantly reduced P300 latency.	Single-blind, nonrandomised, waitlist-controlled pilot trial.
Yamaguchi et al. (2004) [30], Japan	Aim: to test the effects of choto-san on brain function in people with VaD/MCI. Recruitment location: hospital outpatients.	VaD/MCI ($N = 20$): choto-san (8M : 2F, 71.3 ± 9.8 yrs, MMSE 23.8 ± 3.6) or control (9M : 1F, 68.0 ± 8.6 yrs, MMSE 26.8 ± 2.8).	Intervention: 3x choto-san extract (TJ-47, Tsumura, 7.5 g/day) orally, daily [contains 4.5 g of extract of 11 kinds of dried medical herbs: Uncariae Uncis Cum Ramulus (3 g hooks and branch of *Uncaria sinensis* Oliver), Aurantii Nobilis pericarpium (3 g peel of *Citrus unshiu* Markovich), Pinelliae tuber (3 g tuber of *Pinellia ternate* Breitenbach), Ophiopgonis tuber (3 g root of *Ophiopogon japonicus* Ker-Gawler), Hoelen (3 g fungus of *Poria cocos* Wolf), Ginseng radix (2 g root of *Panax ginseng* C.A. Meyer), Chrysanthemi flow (2 g flower of *Chrysanthemum morifolium* Ramatulle), Saphoshnikoviae radix (2 g root and rhizome of *Saposhnikovia divaricata* Schischkin), Glycyrrhizae radix (1 g root of *Glycyrrhiza uralensis* Fisher), Gypsum Fibrosum (5 g $CaSO_4$ $2H_2O$) and Zingiberis rhizoma (1 g, rhizome of *Zingiber officinale* Roscoe)]. Duration: 12 weeks.	ERP (P300 novelty auditory oddball) NTB: MMSE, verbal fluency test.	Cognition: significantly faster RT and increased accuracy on auditory oddball task. Significant improvement in MMSE and verbal fluency. Neuroimaging: Significant reduction in P300 latency. Trend towards increased novelty P300 amplitude.	Open, cohort study.

Table 3: Continued.

Author (reference)	Aim, recruitment	Study population	Intervention and duration	Neuroimaging and neuropsychological measures	Efficacy on cognition, neuroimaging measures, any associations, adherence, retention, and adverse events	Study design
Zhang et al. (2015) [31], China	Aim: to investigate the effect of Bushen capsule (BSC) on brain function in people with aMCI. Recruitment location: hospital and community.	aMCI ($N = 44$): randomised to BSC (12M : 10F, 65.05 ± 6.67 yrs, MMSE 26.27 ± 1.58) or placebo (11M : 11F, 62.41 ± 5.76 yrs, MMSE 26.45 ± 1.40).	Intervention: 4 × 300 mg BSC [main components Zexie (*Alismatis rhizoma*) and Roucongrong (*Cistanches Herba*)] or 4x placebo tablet, 3x/day. Duration: 3 months.	fMRI (episodic memory encoding task). NTB: MMSE, AVLT, CVLT, Stroop, digit symbol, clock drawing.	Cognition: significant improvement in MMSE, stroop, and AVLT. Neuroimaging: increased brain activation in right putamen; this was significantly associated with stroop performance. Reduced brain deactivation in right middle temporal gyrus; this was significantly associated with AVLT performance. Adverse effects: 1x decreased appetite for 3 days (BSC), 1x mild nausea for 1 week (placebo). Neither discontinued use or withdrew from study.	Randomised, double-blind, placebo-controlled trial
Zhang et al. (2014) [32], China	Aim: to investigate the effect of Congrongyizhi capsule (CCRC) on brain function in people with aMCI. Recruitment location: hospital and community.	aMCI ($N = 41$): randomised to CCRC (8M : 8F, 64.25 ± 7.10 yrs, MMSE 26.38 ± 1.50), placebo (4M : 6F, 60.20 ± 3.52 yrs, MMSE 26.70 ± 1.64), or control (6M : 7F, 60.08 ± 6.53 yrs, MMSE 26.77 ± 1.30).	Intervention: 4x CCRC [main components Cistanche and Polygonum multiflorum thunb] or 4x placebo tablet, 3x/day or nothing (control). Duration: 3 months.	fMRI (n-back task). NTB: MMSE, AVLT, CVLT, stroop, digit span, clock drawing.	Cognition: significant improvement in MMSE and digit span, which were significantly associated with increased brain deactivation in posterior cingulate cortex. Neuroimaging: increased brain deactivation in posterior cingulate cortex, inferior frontal gyrus, and lingual gyrus. Adverse effects: 1x decreased appetite for 3 days (CCRC), 1x mild nausea for 1 week (placebo). Neither discontinued use or withdrew from study.	Randomised, double-blind, placebo-controlled trial.
Zhang et al. (2012) [33], China	Aim: to investigate the effect of *Ginkgo biloba* (EGb761) on brain activity in people with VaD. Recruitment location: hospital.	VaD ($N = 80$: 46M : 34F, 66.5 ± 5.6 yrs) randomised to GBT ($n = 40$) or aspirin ($n = 40$).	Intervention: 19.2 mg GBT + 75 mg aspirin or 75 mg aspirin tablet, 3x/day. Duration: 3 months.	rCBF (transcranial Doppler) NBT: Montreal cognitive assessment (MoCA).	Cognition: significant improvement in global score MoCA, as well as MoCA score indices for executive function, attention, delayed memory, and orientation. Neuroimaging: significant increase in blood flow velocity in middle and anterior cerebral arteries.	Randomised, controlled trial.

Note. ACC = anterior cingulate cortex; AD = Alzheimer's disease; ADAS-Cog = Alzheimer's Disease Assessment Scale–cognitive subscale; aMCI = amnestic mild cognitive impairment; AVLT = Auditory Verbal Learning Test; BNT = Boston Naming Test; CGI = clinical global impression; COWAT = Controlled Oral Word Association Test; CVLT = California Verbal Learning Test; d = day (i.e., /d = per day); DMN = default mode networks; DRS = Dementia Rating Scale; EEG = electroencephalography; ERP = event-related potential; FAB = frontal assessment battery; F : M = females to males; fMRI = functional magnetic resonance imaging; GM = grey matter; HVLT-R = Hopkins Verbal Learning Test-Revised; MCI = mild cognitive impairment; MFG = medial frontal gyrus; MID = multi-infarct dementia; MMSE = Mini-Mental State Examination; MoCA = Montreal cognitive assessment; MRI = magnetic resonance imaging; MTG = medial tegmental gyrus; NPI = Neuropsychiatric Inventory; NTB = neuropsychological test battery; OBS = Organic Brain Syndrome Scale; PCC = posterior cingulate cortex; PET = positron emission tomography; PSMS = Physical Self-Maintenance Scale; RAVLT = Rey Auditory Verbal Learning Test; rCBF = regional cerebral blood flow; RCFT = Rey Complex Figure Test; rCMRglc = regional cerebral metabolic rate of glucose consumption; ReHo = regional homogeneity; RT = reaction time; SCAG = Sandoz Clinical Assessment-Geriatric; SDAT = senile dementia of the Alzheimer type; SH = subcortical/periventricular hypertensity; SPECT = single-photon emission computed tomography; tHcy = total homocysteine; TMT = Trail Making Test; VaD = vascular dementia; V-EEG = vigilance-controlled EEG; yrs = years (i.e., age in years); WAIS-R = Wechsler Adult Intelligence Scale–Revised; WMS-R = Wechsler Memory Scale–Revised.

Table 4: Risk of bias ratings for included studies. Studies are detailed in alphabetical order of authors' names. Studies with low risk of bias (total scores ≥ 9) are italicised.

Study	Item 1	Item 2	Item 3	Item 4	Item 5	Item 6	Item 7	Item 8	Item 9	Item 10	Total
Bi et al. (2011)	0	0	1	0	1	1	1	1	0	0	5
Cohen et al. (2003)	0	0	1	0	1	1	1	1	0	0	5
de Waal et al. (2014)	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*10*
Douaud et al. (2013)	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*0*	*9*
Heo et al. (2016)	0	0	1	0	0	0	0	1	1	0	3
Hofferberth (1994)	0	0	0	0	0	0	0	1	0	0	1
Jernerén et al. (2015)	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*0*	*9*
Köbe et al. (2015)	0	0	1	0	0	1	1	1	1	0	5
Matsuoka et al. (2012)	0	0	1	0	1	1	1	1	1	0	6
Muresanu et al. (2010)	0	0	1	0	0	0	1	1	0	0	3
Nilsson et al. (2000)	0	0	1	0	1	1	0	1	0	0	4
Oishi et al. (1998)	0	0	0	0	0	0	0	1	1	0	2
Onofrj et al. (2002)	0	0	1	1	1	0	0	1	1	0	5
Saletu et al. (1995)	0	0	1	0	1	0	1	1	1	1	6
Smith et al. (2010)	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*10*
Thomas et al. (2001)	1	0	0	0	1	1	1	1	0	0	5
Tsolaki et al. (in press)	0	0	0	0	0	0	0	0	0	0	1
Yamaguchi et al. (2004)	0	0	0	0	0	0	0	1	0	0	1
Zhang et al. (2015)	0	0	1	1	0	1	1	1	1	0	6
Zhang et al. (2014)	0	0	1	1	0	1	1	1	1	0	6
Zhang et al. (2012)	1	0	1	0	1	0	0	1	0	0	4

Note. Items rated as yes scored 1, and items rated as no or unable to determine both scored 0. Lower scores indicate a higher risk of bias.

of which 13 were in the form of a tablet/capsule [14, 16–22, 27, 28, 31–33], 3 as a granular or powder extract [13, 23, 30], 1 as a drink [15], and 2 with no details of the preparation method (traditional Chinese medicine [26] and Crocus [29]). One of those studies was a multidomain intervention with omega-3 fatty acid supplementation, aerobic training, and cognitive stimulation [22]. One study gave intramuscular injections [25] and 1 intravenous infusions [24].

3.2.4. Neuroimaging Techniques. Ten studies used EEG [15, 19–21, 24, 26–30], 5 used MRI [14, 16–18, 22], 2 used fMRI [31, 32], 1 used SPECT [23] and another PET [13], and 3 studies measured CBF [25, 26, 33]. One CBF study employed xenon 133 inhalation and high resolution scintillation detectors (Cortexplorer®) [25], 1 used transcranial Doppler (TCD) [33], and the other used stable xenon CT; that study also recorded EEG [26]. One other study combined methods: EEG and MRI [29].

A range of analyses were conducted for EEG, MRI, and fMRI studies. For the EEG studies, 1 examined functional connectivity using phase lag index [15], 2 studies assessed relative power with quantitative EEG (qEEG) from an eyes closed resting state condition [21, 24], 1 study examined theta/alpha ratio [19], and 6 studies assessed P300 ERP component amplitudes and latencies from an auditory oddball task [20, 26–30], 1 of which also analysed N200 [29], and another also employed a 3-minute vigilance task and assessed absolute and relative power [20]. For the MRI studies, 1 examined whole brain volume and subcortical and periventricular hyperintensities [14], 1 regional volumetric changes [29], 2 examined regional grey matter volume [16, 22], and 2 examined whole brain atrophy [17, 18]. Of the 2 fMRI studies, both assessed blood oxygenation level dependent (BOLD) responses, 1 with an episodic memory encoding task [31], and another with an n-back task [32].

3.2.5. Measures of Cognition. A variety of neuropsychological measures were used to assess cognition. The most common were the MMSE ($n = 20$) [13–18, 20–33], Alzheimer's Dementia Assessment Scale-cognitive subscale (ADAS-cog; $n = 4$) [13, 24, 27, 28], Hopkins Verbal Learning Test (HVLT-R; $n = 4$) [16–18], Auditory Verbal Learning Test ($n = 3$) [22, 31, 32], Rey Auditory Verbal Learning Test ($n = 1$) [15], California Verbal Learning Test ($n = 3$) [14, 31, 32], Stroop Test ($n = 3$) [22, 31, 32], Trail Making Test ($n = 3$) [14, 15, 22], Clinical Dementia Rating-Sum of Boxes (CDR-SOB; $n = 3$) [16–18], and Category Fluency ($n = 3$) [16–18]. Please refer to Table 3 for other neuropsychological tests used.

3.2.6. Compliance, Withdrawals, and Adverse Events. Nine studies reported on compliance [15–18, 22–24, 27, 28], 14 reported on withdrawals (loss to follow-up) [13–18, 20, 22–24, 27, 28, 31, 32], and 11 reported on adverse events [13, 16–18, 20, 22, 23, 26, 27, 31, 32]. Information on the reporting of compliance, withdrawals, and adverse events is summarised in Table 3.

3.3. Risk of Bias within and across Studies. Table 4 details the results for the risk of bias assessment (refer to Table 2 for

items assessed). Although 13 studies were randomised [13–18, 20, 24, 27, 28, 31–33], only 5 studies detailed how the randomisation procedure was conducted [15–18, 28], and 4 of those studies reported specific information on the allocation concealment [15–18]. Participant characteristics including how the diagnosis of cognitive decline, MCI, or dementia was made or confirmed, and inclusion and exclusion criteria were described in 16 studies [13–18, 20–25, 27, 31–33]. Seven studies reported on blinding of participants, intervention deliverers, and researchers collecting data [15–18, 27, 31, 32]. Only 12 out of the 21 studies provided sufficient information on the intervention to allow replication [13–18, 20, 23, 25, 27, 28, 33], and only 12 described neuroimaging methodologies and analyses sufficiently to allow replication [13–18, 22, 23, 25, 28, 31, 32]. Thirteen studies used appropriate, valid, and reliable outcome measures [13–18, 20, 22–24, 28, 31, 32]. The majority of studies did not selectively report [13–33]. Adverse events were reported in 12 studies [15–18, 20–23, 26, 27, 31, 32], and only 3 studies reported a power calculation, all of which were sufficiently powered to detect an effect [15, 18, 20].

3.3.1. Intervention Efficacy in Low Risk of Bias Studies. Four of the 21 studies were reported particularly well and demonstrated a low risk of bias (scoring ≥ 9) [15–18]. Three of those studies were reporting on findings from the same randomised, double-blind, placebo-controlled trial (VITACOG) [16–18] investigating the effects of 2 years of high dose vitamin B treatment for people with MCI, and the other was a randomised, double-blind, placebo-controlled 24-week international multisite clinical trial [15] on Souvenaid® for AD. All 4 studies incorporated a relatively comprehensive neuropsychological test battery, rather than just a simple global measure of cognition (e.g., ADAS-cog, MMSE). One of those studies reported on EEG network connectivity [15], and the other 3 reported structural MRI: regional grey matter volume [16] and whole brain atrophy [17, 18]. One study found a reduction in EEG beta network integrity in the placebo, but not the intervention group, indicating counteraction of network decline after 24 weeks of 125 mL/day Souvenaid in people with AD [15]. The other 3 studies showed a reduction in regional grey matter and whole brain atrophy after 2 years treatment with high dose vitamin B (0.8 mg/day folic acid, 20 mg/day vitamin B6, 0.5 mg/day vitamin B12) for people with MCI, compared to placebo [16–18].

Three of the 4 studies reported associations between cognitive test scores and neuroimaging outcome measures [15, 16, 18]. In 1 study, an association between EEG beta activity and memory performance (z-score across NTB; see Table 3) was reported at midpoint in the Souvenaid group only [15]. An association between rate of atrophy and both final MMSE scores and baseline Telephone Interview of Cognitive Status-Modified (TICS-M) scores was reported in one of the high dose vitamin B studies [18]. There was also an association between increased grey matter loss and lower MMSE and CDR-SOB scores and poorer delayed recall and category fluency performance [16] in another of the vitamin B studies.

3.4. Efficacy on Neuroimaging Measures across All Studies. Eighteen studies reported positive neuroimaging findings associated with CM treatment [13, 15–26, 29–33] (8 MCI studies, 6 AD studies, 3 VaD studies, and 1 all-cause dementia study) and three reported negative findings [14, 27, 28] (2 AD studies and 1 VaD study). The key patterns of results are outlined below; for more detailed information on results not reported in the review body, please refer to Table 3.

Out of the 6 studies that assessed auditory oddball P300 ERP component amplitudes and latencies, 4 reported reduced P300 latencies [20, 26, 29, 30] and 2 reported increased P300 amplitudes [29, 30] after CM treatment (12 weeks of traditional Chinese medicine versus no comparison group [26]; 8 weeks of 60 mg/day nicergoline *cf.* placebo [20]; 12 weeks of 7.5 g/day Choto-san extract [TJ-47] versus no treatment [30]; 52 weeks of Crocus extract versus waitlist [29]). Two other studies [27, 28] reported similar changes in the control condition: both reported increased P300 amplitudes (26 weeks of 5 mg/day donepezil [27, 28] or 1.5 mg/day rivastigmine [28]), and 1 reported reduced P300 latencies (26 weeks of 5 mg/day donepezil) [27]. Those two studies also showed a decrease in P300 amplitude and an increase in latency following 26 weeks of 2000 IU/day vitamin E [27, 28]. Theta was significantly reduced in the theta/alpha quotient after 12-weeks treatment with 80 mg/day standardised ginkgo biloba extract (EGb761) in one study [19], and another study reported decreased beta network EEG in the placebo but not the intervention group (24 weeks of 125 mL/day Souvenaid) [15].

One MRI study showed significantly increased whole brain volume after 26 weeks of the target multimodal intervention (see Table 3 for details) and reduced volume for the control group [22], and another study showed no difference in whole brain volume between treatment (52 weeks of 1 g/day citicoline) and placebo [14]. One fMRI study reported both increased BOLD response in the right putamen and reduced BOLD in the right middle temporal gyrus when participants completed an episodic memory encoding task after 1.2 g/day Bushen for 12 weeks [31].

One CBF study reported an increase in white matter CBF with stable xenon CT after 12 weeks of a traditional Chinese medicine [26], and one TCD CBF study reported increased blood flow velocity to the middle and anterior cerebral arteries after 12 weeks of 19.2 mg/day EGb 761 standardised ginkgo extract and 75 mg/day aspirin [33].

3.5. Efficacy on Cognition across All Studies. Across all studies, 13 reported positive effects on cognition [13, 19–21, 23–26, 29–33], 4 studies reported negative results [14, 22, 27, 28], and 4 did not report on cognition findings alone [15–18]. As detailed above in Section 3.4, the key patterns of results for the commonly used neuropsychological tests are detailed below, with further information available in Table 3. Two studies [13, 24] reported improvements (a reduction) in ADAS-cog scores in the intervention group (12 weeks of 10 g/day fuzhisan [13]; 4 weeks of 10 mL/day Cerebrolysin [24]), and 1 in the control group (26 weeks of 10 mg/day donepezil [27]), and another showed a significant deterioration in ADAS-cog scores following treatment in the CM arm (26 weeks of 2000 IU/day Vitamin E) but noted improvements in the other two parallel arms (5 mg/day donepezil and 1.5 mg/day rivastigmine) [28].

Five studies [20, 26, 30–32] reported significantly improved MMSE scores after treatment (12 weeks of a traditional Chinese medicine [26]; 8 weeks of 60 mg/day nicergoline [20]; 12 weeks of 22.5 g/day TJ-47 Choto-san extract [30]; 12 weeks of 1.2 g/day bushen [31]; 12 weeks of 3/day Congrongyizhi capsules [32]) and another showed a trend towards improved MMSE scores following 8 weeks of 7.5 g/day of toki-shakuyaku-san powder [23]. One study reported no changes in cognition following 6 months of a multimodal intervention [22].

3.6. Associations between Neuroimaging and Cognitive Measures. Six of 21 studies reported associations between measures of cognition and neuroimaging markers [15–18, 31, 32]. One study showed a relationship between activation in the right putamen during an episodic memory task and Stroop performance, and reduced middle temporal gyrus deactivation with AVLT performance [31]. Another study showed that greater posterior cingulate cortex deactivation was associated with improved MMSE and digit span scores [32].

Four studies did not report neuropsychological test battery findings alone as they had already been published previously [15–18]. For example, one of those studies reported an association between memory performance and EEG beta band activity at the midpoint of the 24-week Souvenaid trial (125 mL/day) [15]. Please see Table 3 for more detailed information on studies reporting associations between clinical and neuroimaging findings.

4. Discussion

This systematic review summarised and critically appraised intervention studies that incorporated neuroimaging outcome measures to assess nutritional and herbal medicines for MCI and dementia. The majority of studies focused on participants with AD [13, 15, 19, 21, 23, 25–28] or MCI [16, 17, 22, 23, 29, 31, 32], utilised a herbal medicine [13, 19, 21, 23, 26, 29–33], a 12-week long intervention [13, 19, 21, 26, 30–33], and incorporated EEG [15, 19–21, 24, 26–30] or structural MRI [14, 16–18, 22] as a neuroimaging technique. All but 3 studies [14, 27, 28] reported positive neuroimaging results following CM treatment [13, 15–26, 29–33], despite most ($n = 17$) studies having a high risk of bias, scoring ≤ 6 out of 10 on the risk of bias assessment [13, 14, 19–33]. Given the importance of using neuroimaging markers in the assessment of endpoints for clinical trials in dementia [35], particularly with a move towards preclinical disease phases [36], and the viable role that CMs can play as potential treatments, it is imperative that the rigour and quality of CM dementia studies using neuroimaging techniques is improved. This discussion will now focus separately on the three aims of this review: (1) study characteristics; (2) methodologies; and (3) intervention efficacy. To address risk of bias, an additional discussion on study quality has also been included. In light of the findings from this systematic review, a series of key recommendations for improving future work in this area is detailed in Box 1.

4.1. Study Characteristics

4.1.1. Participants. The majority of studies reported information on how a diagnosis of MCI or dementia was made or confirmed and included sufficient inclusion and exclusion criteria to allow replication [13–18, 20–25, 27, 31–33]; one study did not detail cognitive status of the control group [30]. Important demographic information, such as years of education, a factor known to significantly increase the risk of dementia [37], was missing from other studies [19, 28]. In order to meaningfully assess the efficacy of an intervention, it is essential that the tested cohort is as homogeneous as possible. This can be done by closely following guidelines stipulating the most up-to-date diagnostic criteria for MCI [38], dementia (relative to the type; e.g., McKhann et al. [39]), and subjective cognitive complaints [40], and by carefully recording and reporting all relevant participant demographics and baseline characteristics. Care must also be taken to match participant characteristics between active and control groups, with one study not detailing information on the cognitive status of the control group, making comparison impossible [30].

4.1.2. Study Setting. The majority of studies recruited from memory clinics [14, 15, 22, 29], hospitals [13, 23, 33], the community [16–18], or a combination of those settings [31, 32]. However, 5 studies did not report the recruitment setting [19, 24, 26–28]. The recruitment setting for dementia studies has been shown to dramatically influence the participant characteristics and health outcomes. For example, participants with MCI recruited from a memory clinic have been shown to have an annual conversion rate to dementia that is 10% higher than participants recruited from the community [41]. Thus, future work in this field should ensure that the recruitment setting is carefully considered in study design and reported adequately in the published results.

4.1.3. Intervention. The majority of studies tested a Chinese herbal medicine [13, 19, 21, 23, 26, 30–33] or a vitamin [16–18, 25, 27, 28] intervention, with most using a tablet or capsule for oral administration [14, 16–22, 27, 28, 31–33]. Only just over half the studies reported enough detail for the intervention to be replicated [13–18, 20, 23, 25, 27, 28, 33]. The main difficulty here was that, for herbal medicines, standardisation did not occur [26, 30], or the details were not supplied. For the latter, this included missing information on the particular standardised formula used (e.g., EGb 761 for *Ginkgo biloba*), missing information on dose or dosing regimen, and/or inadequate information on commercially available extracts (e.g., brand/manufacturer) [19, 21, 22, 26, 29–32]. Quality control and quality assurance (Good Manufacturing Practice [GMP]) is required for psychopharmacological research, and the absence of complete information on intervention formulation makes results near impossible to replicate. This problem is further compounded when multi-herb formulas are used, as a greater degree of preclinical work is required to develop standard operating procedures (SOPs) for extraction methods, and to optimise ratios of individual constituents. It should also be noted that treatment duration varied

(1) Study Characteristics:
(a) Research needs to follow the most recent guidelines stipulating diagnostic criteria for subjective cognitive complaints, MCI, and dementia, and should be closely adhered to when formulating protocols to ensure that the study population is as homogeneous as possible.
(b) Essential baseline characteristics, particularly ones known to increase the risk of dementia, should be reported.
(c) Recruitment setting needs to be carefully considered and always reported.
(2) Methodologies:
(a) All information on neuroimaging data collection, pre- and post-processing pipelines, and quantification needs to be reported to ensure that the results can be adequately scrutinised and replicated.
(b) Optimal analytic techniques should be utilised for the quantification of neuroimaging data.
(c) Standardised neuropsychological tests that are appropriate clinical trial endpoints for the level of cognitive impairment should be used.
(3) Intervention Efficacy:
(a) Standardised herbal extracts should be used to reduce the variability between studies.
(b) Multi-herb formulas require substantial preclinical research to optimise ratios of active components, and determine their efficacy and safety as a formula.
(c) Dosage should be kept similar to other research (unless there is a rationale for adjusting dose) to reduce variability between studies.
(d) The length of trials should be carefully determined and have a rationale to allow for greater comparability between studies.
(e) An appropriate control group, such as a placebo matched to colour, shape, taste and smell of the active treatment, should always be included.

Box 1: Recommendations for future chronic CM neuroimaging research on people with MCI or dementia.

substantially between studies from 4 weeks [24, 25] to 2 years [16–18], adding further complexity to comparisons between studies.

4.1.4. Study Design. Although the majority of studies included a control group [13–20, 22, 24, 27–33], four studies did not [21, 23, 25, 26], rendering a high risk of bias. A control group, such as a placebo, should always be incorporated to establish whether a true relationship between the treatment and outcome actually exists. In the context of herbal medicine research, appropriate placebos are often difficult to establish because they need to match the active treatment on taste, smell, look, and feel. Herbal medicines can be pungent and have a distinctive taste so additional care needs to be taken when matching to a placebo [42].

4.2. Methodology

4.2.1. Structural and Functional Neuroimaging Methods. Most studies incorporated functional neuroimaging methods [13, 15, 19–21, 23–28, 30–33], largely EEG [15, 19–21, 24, 26–30]. There were large differences in the tasks and analytic methods described in these studies, but the majority of EEG papers assessed auditory oddball P300 ERP component amplitudes and latencies [20, 26–30]. The P300 has been widely explored in ERP literature and has been associated with a range of cognitive processes including memory [43], the orienting of attention [44], decision-making [45], and expectancy [46, 47]. The studies assessing P300 in this review largely reported baseline-to-peak quantification methods (when quantification was described at all), despite this being an ineffective approach for disentangling the multiple subcomponents comprised within the monolithic P300 peak (i.e., P3a, P3b, Novelty P3, and Slow Wave) that represent a range of cognitive processes [48]. Given that effect sizes from CMs can be small [49], and that interventions may affect various cognitive domains, it is imperative that optimal analytic methods are employed to maximise the chance of detecting an effect. Alternative component quantification methods, such as Principal Components Analysis (PCA), should be adopted for future CM ERP studies [50].

Neuroimaging data acquisition, pre- and postprocessing pipelines, and analyses were adequately reported in only 12 of 21 studies [13–18, 22, 23, 25, 28, 31, 32]. There was insufficient information on how the data were collected (e.g., recording parameters, task details including length of resting state condition, and stimulus delivery) [19–21, 24, 26, 30, 33], inadequate reporting of pre- and postprocessing techniques that are in line with widely accepted best practice (e.g., artefact rejection) [21, 29], and missing data quantification details (e.g., Fast Fourier Transformation [FFT] parameters, quantification of P300) [24, 26]. Given the potential limitations of some neuroimaging techniques (as outlined in Table 1), it is imperative that future work describes all data acquisition, processing, and analytic techniques to ensure that variability in results between studies can be adequately accounted for.

Although the majority of studies reported positive results [13, 15–26, 29–33], as noted above, the quality of reporting in most of these studies was relatively poor, indicating a high risk of bias. The results and conclusions from those studies should be viewed with a degree of caution. Given that functional neuroimaging methods are often more sensitive than standard pen-and-paper tests, it is even more important that high quality data, analyses, and interpretations are reported.

4.2.2. Measures of Cognition. The majority of studies utilised the MMSE [13–18, 20–28, 30–33], ADAS-cog [13, 24, 27, 28], and tested verbal learning [14–18, 22, 31, 32]. Similar to the neuroimaging results, most studies reported positive effects on cognition [13, 19–21, 23–26, 30–33], even though the risk of bias assessment indicated that only 13 studies used appropriate outcome measures [13–18, 20, 22–24, 28, 31, 32]. For example, it has been argued that the MMSE is not appropriate for cognitive assessments in people with MCI due to its low sensitivity (18%) in that cohort [51]. However, all but 1 [17] of the 7 MCI studies included here reported MMSE scores. These shortcomings make it challenging to meaningfully interpret the efficacy on cognition of the CMs reviewed here. The 4 studies that scored a low risk of bias utilised comprehensive neuropsychological test batteries [15–18] and did not report on the efficacy of these cognitive outcome measures as they had already been reported previously when the complete results of those RCTs were published elsewhere. Future work should also utilise a comprehensive neuropsychological test battery and use outcome measures that are appropriate clinical trial endpoints for the level of cognitive impairment of the target cohort [52].

4.3. Study Quality and Risk of Bias. The majority of studies assessed in this systematic review were at high risk of bias [13, 14, 19–33]. One of the most common (and significant) issues was that a power calculation was not reported in the majority of studies (Table 4). Most studies had a relatively small sample size and were consequently at risk of Type II error (false negative). The 3 studies that did conduct a power calculation all achieved their recruitment target [15, 18, 20]. Bias also came from a lack of reporting on how randomisation and allocation concealment were carried out. Most studies were randomised trials [13–18, 20, 24, 27, 28, 31–33]; however, only a small number of these actually reported on the randomisation procedure [15–18, 28] and an even smaller number on how allocation was concealed [15–18]. Randomisation allows for the distribution of participant characteristics to be left to chance. Without adequate randomisation, it cannot be assumed that the null hypothesis (that participant groups have been drawn from the same population) is true [53]; this jeopardises internal validity. In relation to allocation concealment, given that most studies utilised an oral intervention, there is no reason that similar future work should not report how allocation was concealed and who was blinded. It must be acknowledged that this is not always the case in some physical activity interventions [22], where allocation concealment can be challenging. A further source of bias came from the lack of reporting of adverse events, which was done by only 12 studies [15–18, 20–23, 26, 27, 31, 32]. Future work should always report adverse events that may have been due to the intervention as it ensures the safety of participants.

4.4. Intervention Efficacy. The focus of the 4 high quality studies that scored a low risk of bias [15–18] was to report detailed analyses of neuroimaging secondary outcome measures. Of those four studies, 3 reported that 2-year treatment for MCI with high dose vitamin B (0.8 mg/day folic acid, 20 mg/day vitamin B6, and 0.5 mg/day vitamin B12) reduced whole brain and regional grey matter atrophy, compared to placebo [16–18], and 1 found that 24 weeks of 125 mL/day Souvenaid maintained EEG beta network integrity in people with AD, where this declined in the placebo group [15].

Three of those studies also reported an association between cognitive test scores and neuroimaging outcome measures [15, 16, 18]. It was found that lower MMSE, CDR-SOB, delayed recall, and category fluency scores were associated with accelerated grey matter loss in one of the high dose vitamin B studies [16]. Baseline TICS-M and final MMSE scores were associated with rate of atrophy in another high dose vitamin B study [18], and midpoint memory performance was associated with beta activity in the Souvenaid study [15]. In terms of clinical use, the above studies indicate that 2 years of high dose vitamin B or 6 months of 125 mL/day Souvenaid have potential clinical utility as an adjunct therapy for people with MCI or Alzheimer's disease, respectively.

4.5. Recommendations. This systematic review has identified a number of consistent shortcomings in CM neuroimaging research into cognitive decline. In an effort to improve the rigour and validity of this important and developing field, the authors suggest 11 key recommendations emerging from the 3 review aims that future work should adhere to. These are detailed in Box 1.

4.6. Strengths and Limitations. This systematic review focused on studies reporting a chronic intervention only. Acute studies may necessarily utilise a different range of neuroimaging methods than those reported here. For example, structural MRI is not appropriate for acute treatment administration as structural brain changes take longer than a few hours to be detected. Future research should systematically summarise and critically appraise acute CM studies [54, 55] to provide a more comprehensive overview of the field. Furthermore, the heterogeneity of the interventions and neuroimaging techniques employed made meta-analyses impossible here. Future work (with a different aim) could consider focusing on only one intervention or neuroimaging modality in order to quantify efficacy. It should also be noted that the authors of included studies were not contacted by the authors of this review.

This review not only focused on efficacy but also on summarising the characteristics of studies, intervention efficacy, and methods utilised. Particular consideration was given to identifying risks of bias. Neuroimaging and CM are a rapidly evolving area of research; thus the findings reported here highlight a number of significant strengths and weaknesses in this field that can be addressed in future work in an effort to improve the evidence base.

4.7. Conclusions. This systematic review summarised and critically appraised CM research on people with cognitive decline, MCI, or dementia that incorporated neuroimaging as an outcome measure. It was found that most studies focused on people with AD, utilised a herbal medicine intervention that was on average 12 weeks long, and used EEG or structural MRI as neuroimaging outcome measures. Nearly all studies

reported positive results, despite the majority having a high risk of bias. The most common issues were a lack of reporting on randomisation, allocation concealment, blinding, and the lack of a power calculation. Eleven recommendations to improve future neuroimaging CM research on people with MCI and dementia have been highlighted in the recommendations box. The authors hope that the pragmatic approach taken to this systematic review will lead to an uptake of these recommendations and a subsequent increase in the quality of CM neuroimaging research on people with MCI or dementia.

Competing Interests

As a medical research institute, NICM receives research grants and donations from foundations, universities, government agencies, individuals and industry. Sponsors and donors provide untied funding for work to advance the vision and mission of the Institute. The project that is the subject of this article was not undertaken as part of a contractual relationship with any organisation other than the funding declared in the Acknowledgements. It should also be noted that NICM conducts clinical trials relevant to this topic area, for which further details can be provided on request.

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Research Article

Medicinal Plants of the Australian Aboriginal Dharawal People Exhibiting Anti-Inflammatory Activity

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Chronic inflammation contributes to multiple ageing-related musculoskeletal and neurodegenerative diseases, cardiovascular diseases, asthma, rheumatoid arthritis, and inflammatory bowel disease. More recently, chronic neuroinflammation has been attributed to Parkinson's and Alzheimer's disease and autism-spectrum and obsessive-compulsive disorders. To date, pharmacotherapy of inflammatory conditions is based mainly on nonsteroidal anti-inflammatory drugs which in contrast to cytokine-suppressive anti-inflammatory drugs do not influence the production of cytokines such as tumour necrosis factor-α or nitric oxide. However, their prolonged use can cause gastrointestinal toxicity and promote adverse events such as high blood pressure, congestive heart failure, and thrombosis. Hence, there is a critical need to develop novel and safer nonsteroidal anti-inflammatory drugs possessing alternate mechanism of action. In this study, plants used by the Dharawal Aboriginal people in Australia for the treatment of inflammatory conditions, for example, asthma, arthritis, rheumatism, fever, oedema, eye inflammation, and inflammation of bladder and related inflammatory diseases, were evaluated for their anti-inflammatory activity in vitro. Ethanolic extracts from 17 *Eucalyptus* spp. (Myrtaceae) were assessed for their capacity to inhibit nitric oxide and tumor necrosis factor-α production in RAW 264.7 macrophages. *Eucalyptus benthamii* showed the most potent nitric oxide inhibitory effect (IC_{50} 5.57 ± 1.4 μg/mL), whilst *E. bosistoana, E. botryoides, E. saligna, E. smithii, E. umbra, and E. viminalis* exhibited nitric oxide inhibition values between 7.58 and 19.77 μg/mL.

1. Introduction

Inflammation is an important biological process and is essential to maintain the body's homeostasis, to fight against pathogens effectively, and to repair the damaged tissue [1]. However when uncontrolled and chronic, inflammation gives rise to a number of (often age related) diseases including asthma, rheumatoid arthritis, inflammatory bowel disease, Crohn's disease, and tendonitis. Furthermore, a chronic inflammatory response with accompanying oxidative stress is a significant force driving the progression of peripheral diseases like atherosclerosis, diabetes, and metabolic syndrome, as well as neurodegenerative diseases such as multiple sclerosis, Parkinson's disease, and Alzheimer's disease [2–5].

While some chronic/remitting neurological diseases, such as multiple sclerosis, have long been recognized as inflammatory, the term "neuroinflammation" is now applied to chronic activation of microglia and astroglia that do not reproduce the classic characteristics of inflammation in the periphery but may cause neurodegeneration [6–8]. Some examples of diseases characterized by neuroinflammation are Alzheimer's disease (AD) and Parkinson's disease and even autism-spectrum and obsessive-compulsive disorders [9–12]. Microglial and astroglial activation, accompanied by increased levels of proinflammatory mediators such as TNF-α, IL-1β and IL-6, prostaglandins, and reactive oxygen and nitrogen species, as well as reactive carbonyl species and advanced glycation end products, is observed in the AD brain at all stages of the disease [13–18]. Genetic and pharmacoepidemiological studies also point to the importance of inflammation in AD. For example, three immune-relevant genes were shown to be associated with an increased risk of

AD; these are CLU (clusterin), CR1 (complement receptor 1), and TREM2 (triggering receptor expressed on myeloid cells 2) [19].

Consequently, targeting chronic neuroinflammation, for example, with plant-derived anti-inflammatory compounds, has been suggested as a promising disease-modifying treatment for many neurodegenerative diseases including AD [12, 20–27].

At present, both steroidal and nonsteroidal anti-inflammatory drugs (NSAIDs) are used to treat inflammation. NSAIDs in particular can cause severe side effects, most importantly gastric ulcers. NSAIDs are specifically designed as inhibitors of cyclooxygenase (COX) enzymes and, in contrast to CSAIDs, do not influence the production of proinflammatory cytokines such as TNF-α or free radicals such as nitric oxide [28]. CSAIDs specifically target p38 MAPK and NF-κB signalling pathways to inhibit cytokine-mediated events with demonstrated efficacy in a range of animal models [29, 30].

Activated inflammatory cells produce a variety of chemokines and cytokines, reactive oxygen species (ROS), reactive nitrogen species (RNS), free radicals, and prostaglandins [31–33] and cease to produce neuroprotective factors such as glutathione [6, 7].

Excessive production of inflammatory cytokines and reactive radical species can damage cellular biomolecules like proteins, lipids, and carbohydrates as well as nucleic acids, leading to cellular and tissue damage, which further perpetuates the inflammatory cascade. Therefore, pharmacological compounds with the ability to attenuate the production of these inflammatory molecules may have potential for the treatment of many inflammatory diseases including AD [21, 22, 28, 34, 35].

The use of natural substances, especially those derived from plants, in order to prevent, manage, or cure diseases is a centuries-old practice which has led to the discovery of many modern pharmaceuticals. In recent years, the search for novel anti-inflammatory drugs from a wide range of medicinal plant resources has been intensified, and a variety of plant secondary metabolites including apigenin, curcumin, cinnamaldehyde, and resveratrol have already been found to suppress inflammatory responses [21, 22, 28].

For example, turmeric (*Curcuma longa*) and its main ingredient curcumin, which has long been used for treatment of rheumatic disorders, exerts both anti-inflammatory and antiatherosclerotic effects [23, 36]. Ginger extract (*Zingiber zerumbet*) and its main active compound, 3-*O*-methyl kaempferol, significantly attenuated carrageenan-induced mouse paw oedema in an in vivo model and were also found to inhibit the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2), as well as iNOS expression in a cell culture model. Aqueous and hydroalcoholic as well as ethanolic extracts from another ginger species (*Zingiber officinale*) demonstrated significant anti-inflammatory activity and its active constituent [6] gingerol again showed anti-inflammatory activity by inhibiting the production of NO and PGE_2 [37] and was also successful in inhibiting carrageenan-induced rat paw oedema [38].

Triterpenoid saponins, from the Australian desert tree *Acacia victoriae,* have shown anti-inflammatory effects *via* inhibiting activation of NF-κB, by preventing its nuclear localization and inhibiting its ability to bind to DNA [39]. Another Australian indigenous plant *Tinospora smilacina* is claimed to possess long chain unsaturated fatty acids which possess anti-inflammatory properties [40]. The fruits of the Australian native Kakadu plum (*Terminalia ferdinandiana*), Illawarra plum (*Podocarpus elatus*), and Native currant (*Acrotriche depressa*) also exhibited significant anti-inflammatory activity [41].

There is large scope to investigate Australian native plants for their bioactivity and chemical constituents [42]. Traditional medicine is still practised by the many tribal Aboriginal people, particularly in Central and Northern Australia and this ethnomedicinal knowledge is recorded in some cases [43]. The "Dharawal Pharmacopeia" written by botanist and Aboriginal Elder Frances Bodkin (known as Aunty Fran) is a compilation of the Aboriginal medicinal and ceremonial uses (and corresponding taxonomic identification) of thousands of native Australian plants. Of interest to our research, a number of plant species described in the Dharawal pharmacopeia have been claimed to possess anti-inflammatory activities (Table 1) [44, 45]. Plants from *Eucalyptus* species have special importance for the Dharawal indigenous people and are used for their anti-inflammatory activity along with other medicinal uses as well as for shelter and weapons. As stated in the Dharawal pharmacopeia, Eucalypts are mostly distributed in Blue Mountains, Southern Highlands, Woronora Plateau, and coastal area of New South Wales, Australia.

The aim of our research is to evaluate the anti-inflammatory activity of Australian native plants with ethnopharmacological importance and subsequently characterise the bioactive components. In this manuscript, dried extracts from 17 *Eucalyptus* spp. were evaluated for anti-inflammatory activity *via* the suppression of NO and TNF-α production induced by lipopolysaccharide (LPS) and interferon gamma (IFN-γ) in RAW 264.7 cells. Cytotoxicity of the crude extracts was also examined using an Alamar blue cell viability assay.

2. Materials and Methods

2.1. Plant Material. Plants known to be used by the Dharawal people (also known as Tharawal) to treat inflammation and related illnesses were selected under the guidance of botanist and Aboriginal Elder Auntie Fran (Frances Bodkin) and the Dharawal pharmacopeia. Leaf material of 17 *Eucalyptus* spp. was collected in the month of August, 2015 from the "Australian Botanic Gardens" at Mount Annan, NSW, Australia (Table 1).

2.2. Chemicals and Reagents. Ethanol was purchased from Chem-Supply (Gillman, SA, Australia); bovine serum albumin, lipopolysaccharide (*E. coli* serotype-0127:B8), EDTA, *N*-(1-napthyl) ethylenediamine dihydrochloride, benzylpenicillin G sodium salt, resazurin sodium salt (10%), streptomycin, sulphanilamide, 3,3′,5,5′-tetramethylbenzidine (TMB), trypan blue, and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma-Aldrich (Castle Hill,

Table 1: Plants collected for the study of anti-inflammatory activity.

Number	Plant	APNI name	Family	Voucher number
(1)	*Eucalyptus acmenoides*	*Eucalyptus acmenoides* Schauer	Myrtaceae	961604
(2)	*Eucalyptus benthamii*	*Eucalyptus benthamii* Maiden & Cambage	Myrtaceae	832452
(3)	*Eucalyptus bosistoana*	*Eucalyptus bosistoana* F. Muell.	Myrtaceae	20070782
(4)	*Eucalyptus botryoides*	*Eucalyptus botryoides* Sm.	Myrtaceae	861776
(5)	*Eucalyptus eximia*	*Eucalyptus eximia* Schauer	Myrtaceae	841857
(6)	*Eucalyptus globoidea*	*Eucalyptus globoidea* Blakely	Myrtaceae	873240
(7)	*Eucalyptus gummifera*	*Eucalyptus gummifera* (Gaertn.) Hochr.	Myrtaceae	892074
(8)	*Eucalyptus maculata*	*Eucalyptus maculata* Hook.	Myrtaceae	20070782
(9)	*Eucalyptus notabilis*	*Eucalyptus notabilis* Maiden	Myrtaceae	20020217
(10)	*Eucalyptus paniculata*	*Eucalyptus paniculata* Sm.	Myrtaceae	840775
(11)	*Eucalyptus pilularis*	*Eucalyptus pilularis* Sm.	Myrtaceae	861796
(12)	*Eucalyptus punctata*	*Eucalyptus punctata* DC.	Myrtaceae	861820
(13)	*Eucalyptus resinifera*	*Eucalyptus resinifera* Sm.	Myrtaceae	911862
(14)	*Eucalyptus saligna*	*Eucalyptus saligna* Sm.	Myrtaceae	872719
(15)	*Eucalyptus smithii*	*Eucalyptus smithii* R. T. Baker	Myrtaceae	361827
(16)	*Eucalyptus umbra*	*Eucalyptus umbra* R. T. Baker	Myrtaceae	900782
(17)	*Eucalyptus viminalis*	*Eucalyptus viminalis* Labill.	Myrtaceae	861830

NSW, Australia). GIBCO, fetal bovine serum (FBS), and glutamine were purchased from Life Technologies (Mulgrave, VIC, Australia). Murine interferon-γ (IFN-γ) and TNF-α ELISA kits were purchased from PeproTech Asia (Rehovot, Israel). Citric acid and monosodium dihydrogen carbonate (NaH_2CO_3) were from AJAX Chemicals (Auburn, NSW, Australia). Tween-20 was from Amresco (Solon, Ohio, USA). Methanol, monosodium phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4), sodium chloride (NaCl), and sulfuric acid (H_2SO_4) were from Merck (Darmstadt, Germany). Sodium carbonate (Na_2CO_3) was BDH brand supplied by Merck Pty. Ltd. (Kilsyth, VIC, Australia).

2.3. Extraction of Plants Leaves for Biological Assays and HPLC and MS Analysis. Approximately 40 g of fresh leaf material from each plant was extracted using absolute ethanol. The leaves were first cut into small pieces with scissors and then ground to a coarse powder using a hand blender. The coarse powder was filled into the thimbles of an accelerated solvent extraction system (Buchi B-811, Switzerland) and then extracted under standard soxhlet mode (for 2 × 15 minutes cycles). The volume of the extracts was reduced to ca. 2–4 mL using a rotary evaporator and then evaporated to dryness with nitrogen gas for biological assays. Percentage yields (g/g% fresh weight) are recorded in Table 2.

2.4. Maintenance and Preparation of RAW 264.7 Macrophages. RAW 264.7 macrophages were grown in 175 cm^2 culture flasks on DMEM (Dulbecco's Modified Eagle's Medium) containing 5% FBS (fetal bovine serum) that was supplemented with antibiotics (1%) and glutamine (1%). The cell line was maintained in 5% CO_2 at 37°C, with media being replaced every 3-4 days. Once cells had grown to confluence in the culture flask, they were removed using a rubber policeman cell scraper, as opposed to using trypsin, which can remove membrane-bound receptors such as RAGE. The cell suspension was concentrated by centrifugation for 3 min at 900 rpm and resuspended in a small volume of fresh DMEM (with 1% antibiotics and 5% FBS). Cell densities were estimated using a Neubauer counting chamber. Cell concentration was adjusted with DMEM (with 1% antibiotics and 5% FBS) to obtain 60000 cells/100 μL cell suspension. The 100 μL cell suspension was then dispensed into the inner wells of 96-well plates. Plates were incubated at 37°C and 5% CO_2 for 18 h before the activation experiments were carried out.

2.5. Activation of RAW 264.7 Macrophages. From each well, the media were removed and replaced with fresh DMEM containing 0.1% FBS. For assays with extracts, a 90 μL volume of the dilutions in DMEM (with 0.1% FBS) was added an hour prior to addition of the activator. Due to the often inconsistent nature of LPS at activating cells, a combination of LPS (10 μg/mL) and IFN-γ (10 U/mL), both in DMEM (with 0.1% FBS), was used for activation. A maximum dose of the extracts used is 900 μg/mL and diluted serially by 50% up to a minimum of 10 doses (900, 450, 225, 112.5, 56.25, 28.125, 14.062, 7.031, 3.515, 1.7578, and 0.8789 μg/mL in the wells, resp.). After activation, the cells were incubated for 24 h at 37°C and 5% CO_2 and then NO and TNF-α inhibition and cell viability were determined. Cells with media alone were used as negative control and activated cells used as positive control.

2.6. Determination of Nitric Oxide Production by Griess Assay. Nitric oxide was determined by Griess reagent quantification of nitrite, one of its stable reaction products. Griess reagent was freshly made up of equal volumes of 1% sulfanilamide and 0.1% naphthylethylene-diamine in 5% HCl. In the presence of nitrite this reagent forms a violet colour. From each well, 50 μL of supernatant was transferred to a fresh 96-well plate and mixed with 50 μL of Griess reagent, and the colour produced was measured at 540 nm in a microplate

Table 2: Plant common names, ethnomedicine, and yields of ethanolic extracts for the study of anti-inflammatory activity.

Plant species	Common name(s)	Diseases treated using leaves (according to Dharawal Aboriginal medicinal use)	Yield of ethanol extract (%)
Eucalyptus acmenoides	White mahogany/yellow stringybark	Breathing difficulties, chest and muscle pain, fever, and wash for joints	19.2
Eucalyptus benthamii	Camden white gum	Colds, fever, chest and muscle pain, and wash for joints	12.6
Eucalyptus bosistoana	Coastal grey box	Colds, fever, chest and muscle pain, and wash for joints	11.6
Eucalyptus botryoides	Bangalay/southern mahogany	Colds, fever, chest and muscle pain, and wash for joints	41.6
Eucalyptus eximia	Yellow bloodwood	Colds, fever, chest and muscle pain, wash for joints, extreme diarrhea, and syphilitic sores	25.8
Eucalyptus globoidea	White stringybark	Breathing difficulties, chest and muscle pain, fever, and wash for joints	26.2
Eucalyptus gummifera	Red bloodwood/bloodwood	Colds, fever, chest and muscle pain, and wash for joints	15.4
Eucalyptus maculata	Spotted gum	Asthma, colds, fever, chest and muscle pain, and wash for joints	12.6
Eucalyptus notabilis	Mountain mahogany	Colds, fever, chest and muscle pain, wash for joints, and extreme diarrhea	11.0
Eucalyptus paniculata	Grey ironbark	Asthma, morning sickness	16.4
Eucalyptus pilularis	Blackbutt	Colds, fever, chest and muscle pain, and wash for joints	23.2
Eucalyptus punctata	Grey gum	Breathing difficulties, stomach upset, and morning sickness	14.0
Eucalyptus resinifera	Red mahogany	Colds, fever, chest and muscle pain, wash for joints, and extreme diarrhea	12.0
Eucalyptus saligna	Sydney blue gum	Colds, fever, chest and muscle pain, and wash for joints	10.8
Eucalyptus smithii	Gully gum/blackbutt peppermint	Colds, fever, chest and muscle pain, and wash for joints	13.3
Eucalyptus umbra	Broad leafed white mahogany/white mahogany	Colds, fever, chest and muscle pain, wash for joints, and extreme diarrhea	10.5
Eucalyptus viminalis	Manna gum/ribbon gum/white gum	Colds, fever, chest and muscle pain, and wash for joints	19.0

reader (Bio-Rad, Australia). The remaining supernatant from each well was used for a TNF-α assay using commercial sandwich ELISA development kits (catalog number: 900-K54; PeproTech, USA).

2.7. Determination of Cell Viability by Alamar Blue Assay. The Alamar Blue assay is a colorimetric assay involving the cellular reduction of resazurin to resorufin. Alamar Blue solution [100 μL of 10% Alamar Blue (resazurin) in DMEM medium] was added to each well and incubated at 37°C for 1-2 h. After incubation, fluorescence was measured (excitation at 530 nm and emission at 590 nm) using a POLARstar Omega microplate reader (BMG Labtech, Mornington, Australia) and expressed as a percentage of that in control wells after background fluorescence was subtracted.

2.8. TNF-α Determination by ELISA. The supernatants obtained from each well (remaining supernatant after 24 hours of activation) were diluted 30 times using diluent (0.1% w/v bovine serum albumin and 0.05% v/v tween-20 in PBS [1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, and 154 mM NaCl; pH 7.4]) and were used for determination of TNF-α using a commercial sandwich ELISA (catalog number: 900-K54; Peprotech, USA) according to the manufacturer's protocol. Capture antibody was used at a concentration of 1.25 μg/mL in PBS. To make a standard curve TNF-α (10 ng/mL standard) was diluted serially by 50% up to a minimum of 10 doses (10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.019, and 0.0097 ng/mL in the wells, resp.) and was used as the internal standard. TNF-α was detected with a biotinylated second antibody and an avidin peroxidase conjugate with TMB as detection reagent. After ~30 min, the reaction was stopped using 0.5 M sulfuric acid, and the absorbance was measured at 450 nm of measurement filter with a 655 nm of reference filter. The absorbance data was expressed as a percentage of that in control wells after conversion of the concentrations by using a standard curve constructed with defined concentrations of TNF-α. Curve fitting of this standard curve and extrapolation of experimental data were performed using nonlinear regression analysis.

2.9. Data Presentation and Analysis. As the experiments were done in triplicates, the results were expressed as the mean ± SEM. In addition, linear relationships and significance tests of these data sets were also conducted. GraphPad Prism version 6.01 (GraphPad Software Incorporated, USA) was used for growth curve analysis in dose-dependent experiments and to determine the IC_{50} values for NO and TNF-α inhibition as well as LC_{50}.

3. Results and Discussion

In this study, leaves from 17 different *Eucalyptus* spp. were collected in the month of August, 2015. Approximately 40 g of leaves from each of *Eucalyptus acmenoides, E. benthamii, E. bosistoana, E. botryoides, E. eximia, E. globoidea, E. gummifera, E. maculate, E. notabilis, E. paniculata, E. pilularis, E. punctate, E. resinifera, E. saligna, E. smithii, E. umbra, and E. viminalis* were extracted using absolute ethanol (Table 2).

The RAW 264.7 murine macrophages release NO and TNF-α when exposed to bacterial LPS and IFN-γ and on this principle, has become an established experimental model to evaluate in vitro anti-inflammatory activity of extracts [28]. For the purpose of interpretation, the IC_{50} values of NO inhibition are divided into three groups: extracts with IC_{50} < 20 μg/mL are considered as highly potent extracts; a value between 21 and 80 μg/mL is considered as moderately potent, and an IC_{50} < 80 μg/mL is considered as an extract with low potency.

The highest concentration of ethanolic crude extract tested in the anti-inflammatory assay was 900 μg/mL with 0.5-fold serial dilutions. *Eucalyptus benthamii, E. bosistoana, E. botryoides, E. saligna, E. smithii, E. umbra, and E. viminalis* leaf extracts showed the highest activity for NO inhibition with IC_{50} values of 5.57, 7.58, 16.65, 19.77, 17.62, 17.69, and 8.0 μg/mL, respectively (Table 3, Suppl. Figure 1). The extracts from *Eucalyptus acmenoides, E. eximia, E. notabilis,* and *E. pilularis* showed moderate inhibition of NO with IC_{50} values of 56.93, 34.14, 53.84, and 76.17 μg/mL, respectively. Six other species, *E. globoidea, E. gummifera*, *E. maculata*, *E. paniculata, E. punctata, and E. resinifera*, presented low inhibition of NO with IC_{50} values of 82.9, 108.17, 99.94, 130.7, 120.4, and 81.21 μg/mL, respectively (Suppl. Figure 1).

The plant extracts also showed promising TNF-α inhibitory activity (Table 3) with IC_{50} values of 2.06, 8.53, 19.02, 3.41, 2.41, 10.2, and 16.68 μg/mL for *E. benthamii, E. bosistoana, E. botryoides, E. saligna, E. smithii, E. umbra, and E. viminalis*, respectively, which are the same plants in our highly potent NO inhibitor group. On the other hand, the moderately potent extracts from *E. acmenoides, E. eximia, E. notabilis, and E pilularis* showed TNF-α IC_{50} values of 16.53, 4.82, 27.48, and 21.09 μg/mL, respectively (Suppl. Figure 1), whereas extracts from *E. globoidea, E. gummifera, E. maculata, E. paniculata, E. punctata, and E. resinifera* exhibited comparatively lower inhibition of TNF-α production with IC_{50} values of 50.73, 82.73, 136.34, 334.86, 115.73, and 62.11 μg/mL, respectively, which are the plants in our low potency group (Suppl. Figure 1).

The use of Alamar Blue (resazurin) to measure cytotoxicity is an established technique [46]. The results of cytotoxicity (LD_{50}) of our leaf extracts are shown in Table 3. The plants of our highly potent group were also relatively toxic with LC_{50} values of 22.34, 37.17, 108.40, 101.01, 38.96, 236.5, and 31.92 for *E. benthamii, E. bosistoana, E. botryoides, E. saligna, E. smithii, E. umbra*, and *E. viminalis*, respectively, whereas, plants of the lower potency group showed lower toxicity with higher LD_{50} values of 464.74, 313.45, 540.46, 268.59, 522.84, and 268.59 for *E. globoidea, E. gummifera*, *E. maculata*, *E. paniculata, E. punctata*, *and E. resinifera*, respectively. Plants with moderate potency showed a wide range of cytotoxicity with LD_{50} values of 296.22, 64.14, 332.44, and 374.74 for *E. acmenoides, E. eximia, E. notabilis,* and *E. pilularis*, respectively (Suppl. Figure 1).

In future experiments, we will purify the most potent extracts to identify the most active compounds. One major candidate for carrying the anti-inflammatory activity could be 1,8- cineole, the major monoterpene of eucalyptus oil, as it can represent between 60 and 80% of the volatile oils

Table 3: Anti-inflammatory activity and toxicity of extracts determined in RAW 264.7 macrophages.

Plant species	Inhibition of NO production (IC_{50} in μg/mL)	Inhibition of TNF-α production (IC_{50} in μg/mL)	Cytotoxicity (LC_{50} in μg/mL)
Eucalyptus acmenoides	56.93 ± 11.8	16.53 ± 5.9	296.22 ± 189.3
Eucalyptus benthamii	5.57 ± 1.4	2.06 ± 0.7	22.34 ± 9.3
Eucalyptus bosistoana	7.58 ± 1.2	8.53 ± 3.4	37.17 ± 15.6
Eucalyptus botryoides	16.65 ± 2.2	19.02 ± 5.4	108.40 ± 44.9
Eucalyptus eximia	34.14 ± 7.1	4.82 ± 1.6	64.14 ± 23.6
Eucalyptus globoidea	82.9 ± 12.5	50.73 ± 24.0	464.74 ± 199.7
Eucalyptus gummifera	108.17 ± 10.5	82.73 ± 52.3	313.45 ± 125.9
Eucalyptus maculata	99.94 ± 12.1	136.34 ± 78.8	110.22 ± 41.1
Eucalyptus notabilis	53.84 ± 7.7	27.48 ± 14.9	332.44 ± 107.5
Eucalyptus paniculata	130.7 ± 11.6	334.86 ± 192.7	540.46 ± 172.5
Eucalyptus pilularis	76.17 ± 10.3	21.09 ± 9.7	374.74 ± 190.7
Eucalyptus punctata	120.4 ± 15.9	115.73 ± 58.4	522.84 ± 221.4
Eucalyptus resinifera	81.21 ± 13.4	62.11 ± 36.0	268.59 ± 131.6
Eucalyptus saligna	19.77 ± 2.3	3.41 ± 1.3	101.01 ± 36.8
Eucalyptus smithii	17.62 ± 3.5	2.41 ± 1.1	38.96 ± 14.1
Eucalyptus umbra	17.69 ± 2.3	10.2 ± 4.5	236.5 ± 144.3
Eucalyptus viminalis	8.0 ± 1.2	16.68 ± 9.9	31.92 ± 11.9

Note. Results represent the mean ± SEM of 3 experiments in triplicate for NO production and cytotoxicity whereas for TNF-α production it is 1 experiment in triplicate.

derived from eucalyptus leaves depending on the species. Therapeutic concentrations of 1,8-cineol (1.5 μg/mL = 10^{-5}M) inhibited significantly cytokine production in lymphocytes and monocytes [47, 48]. It has to be noted that 1,8-cineol has already gained market acceptance for its anti-inflammatory properties in mouthwashes and cough suppressants or anti-asthmatic medications [48, 49].

The plants studied here were chosen on the basis of their traditional use to treat inflammatory conditions by the Dharawal people of the Campbelltown region (South-west Sydney Australia). All of the plants showed anti-inflammatory activity and demonstrated inhibitory effect on downregulation of NO and TNF-α production with varying potencies, which supports their use in traditional Aboriginal medicine. The content of the anti-inflammatory compounds in the plants, according to traditional knowledge, is also dependent on the plant's environment. In Dharawal country, what is most important when seeking particular medicines from plants is where the plant is growing, that is, not so much the soils, but the other plants that are growing around the particular plant required. For instance, with the Eucalypts, close proximity of an Ironbark (Muggago) and a Ribbon bark (Kai'yeroo) is needed for the anti-inflammatory medicine from the Burringoa (*Eucalyptus tereticornis*) to be most effective. As another example, the Ironbark itself does not need other Eucalypts close by, but it does need the Einadia (one of the saltbushes) to be growing at its base. In addition, if it had been struck by lightning (and this can be confirmed by a line of interrupted bark running from the top of the tree almost to its base), then the anti-inflammatory medicine would be most effective, when using the leaves of the Eucalypts as medicine, the leaves of the trees younger than 7 years were placed on a low fire and the smoke inhaled. However, when the tree is bearing the mature leaves, the leaves were collected and boiled then allowed to cool before being rubbed on the affected part of the body, depending on the species. For the present screening study, the plant material was provided by the Botanical Gardens from random trees in the garden, but for future studies, we will investigate if collection practice based on Dharawal knowledge will improve the inherent activity and/or yield of the anti-inflammatory compounds.

4. Conclusions

The present study suggests that most of the *Eucalyptus* spp. potentially possess interesting anti-inflammatory compounds with low toxicity and the in vitro activity appears to support the traditional use. *Eucalyptus benthamii, E. bosistoana, E. botryoides, E. saligna, E. smithii, E. umbra, and E. viminalis* leaf extracts exhibited strong anti-inflammatory activity by inhibiting NO and TNF-α production in LPS and INF-γ stimulated RAW 264.7 macrophages. Purification and structure identification of the most these extracts are currently underway.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Herbal Medicine for the Treatment of Vascular Dementia: An Overview of Scientific Evidence

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Dementia is a leading cause of mental and physical disability. Vascular dementia (VaD) is the second most common cause of dementia after Alzheimer's disease (AD) constituting 10–15% of the dementia population. Currently there are no approved pharmaceutical options for VaD and the conventional anti-AD therapies provide only modest, short-term relief of symptoms associated with VaD. Herbal medicines have been used for the management of dementia-like symptoms for centuries and may provide viable therapies for VaD due to their multicomponent and multitarget approach. This review is designed to provide an updated overview on the current status of herbal medicine research, with an emphasis on Chinese herbal medicine, for the treatment of VaD or dementia. A case study is also provided to demonstrate the development process of a novel standardized complex herbal formulation for VaD. The article reveals some preliminary evidence to support the use of single and complex herbal preparations for VaD and dementia. Multiple issues in relation to clinical and preclinical research have been identified and future research directions are discussed.

1. Introduction

Dementia is a syndrome associated with progressive impairments in memory and learning ability, cognitive skills, behaviour, activities of daily living, and quality of life. There are more than 47.5 million people with dementia worldwide and 7.7 million new cases are added to the dementia pool each year [1]. In Australia, there are over 353,800 people living with dementia and the number is estimated to increase to 900,000 by the year of 2050 [2]. Dementia has surpassed cerebrovascular disease and lung cancer to become the 2nd leading cause of death in Australia [3].

There are numerous types of dementia, among which vascular dementia (VaD) is the second most common cause after Alzheimer's disease (AD). Other common forms of dementia include Parkinson's disease, dementia with Lewy bodies, frontotemporal dementia, Huntington's disease, and alcohol-related dementia. VaD is associated with cerebrovascular and cardiovascular diseases and constitutes 10–15% of dementia cases in western countries. In developing countries, the prevalence of VaD is higher, accounting for around 30% of the dementia prevalence, which is partially due to poorer control of cardiovascular risk factors [4]. VaD often coexists with other forms of dementia especially AD. Indeed, postmortem studies reveal that over 40% of clinically diagnosed VaD cases also have AD type of neurodegenerative pathology, which is the most common type of mixed dementia [5].

Currently cholinesterase inhibitors and glutamate receptor antagonists are the most effective pharmaceutical options for the treatment of AD [6]. These medications have also been used off-label in some countries for the symptomatic relief in people with VaD, but the safety and the long-term therapeutic benefits of these interventions in VaD remain uncertain.

In the absence of satisfactory pharmacological therapies, many people with VaD or dementia and their carers turn to complementary medicine. The common complementary medicine interventions for VaD and dementia and dementia risk-reduction include herbal medicine, acupuncture,

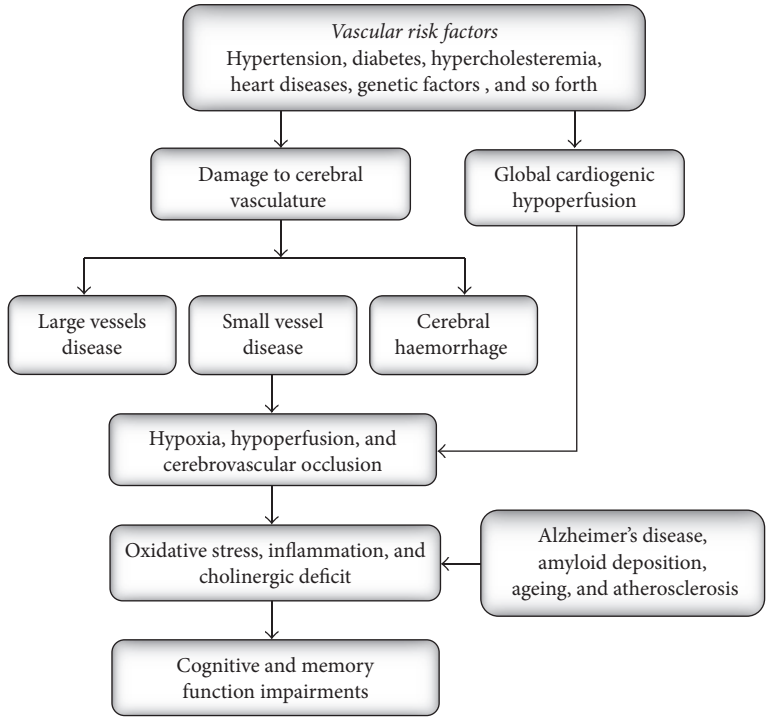

Figure 1: Pathophysiological mechanisms for vascular dementia.

nutraceuticals, yoga, tai chi, and music therapy. The use of herbal medicine for the treatment of ageing-related disorders was documented in the literature more than 2000 years ago in ancient China where herbal remedies were used to boost memory function and increase longevity [7]. Early preclinical and clinical evidence exists to support the use of herbal medicines either as single herbal preparations or as complex herbal formulations for VaD. This review paper aims to provide an updated overview of evidence to support some of the commonly used herbs and herbal combinations with an emphasis on Chinese herbal medicine for the treatment of the disease. Issues and challenges associated with herbal medicines are discussed, and a case study is provided to demonstrate the development process of a novel complex herbal formulation for VaD that takes advantage of modern pharmaceutical and pharmacological technologies.

2. Pathophysiology and Therapeutic Options for Vascular Dementia

Cognitive impairment (especially executive dysfunction) is the primary symptom of VaD, which can also cause a disturbance in mood and behaviour and reduce of quality of life. According to the blood vessels involved and the pathological processes, VaD can be divided into large vessel dementia (multiple infarcts or multi-infarct dementia), small vessel dementia (small vessel disease and microinfarction), strategic infarct dementia, hypoperfusive dementia, dementia related to angiopathies (hypertension, amyloid), haemorrhagic dementia, and familial vascular dementia.

The main risk factors associated with VaD include hypertension, hyperlipidemia, diabetes, genetic disposition, cardiac diseases, physical inactivity, and obesity [8]. The pathophysiology of VaD is complex. It incorporates interactions between vascular aetiologies (cerebrovascular disorders and vascular factors), changes in the brain (infarcts, white matter lesions, and atrophy), and host factors (age, education) [9]. The final common aetiopathogenic pathway usually attributes to a hypoxic, hypoperfusive, or occlusive process resulting in ischemic damage in various areas of the brain with subsequent cognitive and memory function impairments (Figure 1) [10]. Other pathogenic factors such as AD, amyloid deposition, ageing, and atherosclerosis also contribute to VaD development *via* inflammation and oxidative stress [8].

Currently, effective pharmaceutical interventions for VaD are lacking. Standard treatment largely focuses on symptomatic management and prevention of additional brain damage via recognition and control of cardiovascular and cerebrovascular risks using, for example, antihypertensives, aspirin, statins, vascular care, antidiabetes, and lifestyle modification [11]. Several classes of anti-AD pharmaceutical agents are used off-label for symptomatic management in VaD. Cholinesterase (ChE) inhibitors (donepezil, galantamine, and rivastigmine) and NMDA receptor antagonists (memantine) have shown some modest short-term clinical benefits in improving cognitive function; however, most of these studies fail to demonstrate significant improvements in global functioning, activities of daily living, and quality of life [12]. The majority of studies conducted so far are over a relatively short duration (5-6 months); therefore the long-term benefits

and safety of these interventions in VaD have not been validated.

3. Individual Herbs Used in VaD

There is a long history of herbal medicine use to boost memory and cognitive functions and manage behavioral and psychological symptoms associated with dementia/VaD. Some of the most commonly used and studied herbs include *Ginkgo biloba*, *Huperzia serrata*, *Curcuma longa*, *Panax ginseng, Panax notoginseng, Bacopa monnieri, Salvia miltiorrhiza, Crocus sativus,* and *Camellia sinensis*. Table 1 summarises the nomenclature, key bioactive compounds, and mechanisms of action of these herbs.

3.1. Gingko biloba. *Ginkgo biloba* leaf extract (ginkgo) is one of the most studied medicinal herbs. Ginkgo leaf extract is widely used for ageing-related memory disorders in many European and Asian countries. The principal constituents of ginkgo include flavonol glycosides (e.g., quercetin and kaempferol) and terpenoids (e.g., ginkgolide and bilobalide) [13]. Preclinical studies suggest that ginkgo decreases oxygen radical discharge and proinflammatory functions of macrophages (antioxidant and anti-inflammatory), reduces corticosteroid production (antianxiety), and increases glucose uptake and utilisation and adenosine triphosphate (ATP) production [14]. Ginkgo also appears to improve blood flow through increasing red blood cell deformability and decreasing red cell aggregation, inducing nitric oxide production, and antagonising platelet activating factor receptors [14]. EGb761 (a standard ginkgo preparation) treatment was also shown to enhance proliferation of neural stem cells in subventricular zones and the dentate gyrus [15] and to accelerate the recovery of the pathological synaptic plasticity [16] in VaD models in rats. In an ageing rat model, EGb761 reduced circulating free cholesterol and brain β-amyloid precursor protein production [17].

In animal studies, the effects of ginkgo leaf extracts on neuroprotection and cognitive dysfunctions have been demonstrated in various cerebral ischemia models in rats [18, 19], mice [20], and gerbils [21, 22]. In a recent study in rats with two-vessel (bilateral common carotid arteries) occlusion-induced VaD model, treatment of bilobalide significantly improved the learning and memory ability of the animals in a Morris water maze task [23].

In healthy young adults, ginkgo treatment has been shown to improve speed of processing, working memory, executive function, and cognition [24]. In a study with 80 patients with vascular cognitive impairment (not diagnosed with dementia), a combined therapy of ginkgo extract with conventional treatment of aspirin over three months significantly improved executive function, attention, abstract-thinking, delayed memory, and orientation when compared with the control group (aspirin treatment only) [25].

The evidence to support the use of gingko for dementia remains controversial. Although some clinical studies fail to show a significant difference between ginkgo and placebo in dementia groups [26], numerous clinical trials demonstrate that ginkgo improves memory loss and concentration and decreases anxiety in patients with dementia and/or VaD. For example, a randomised, double blind, placebo-controlled trial of 216 participants with AD or vascular dementia showed a significant improvement in attention and memory function in the EGb761-treated group after 24-week treatment [27]. In a more recent trial, 404 people with dementia (333 AD and/or mixed dementia and 71 VaD) were treated with 240 mg EGb 761 or placebo over 24 weeks. The results demonstrate that gingko treatment significantly improves cognitive function and neuropsychiatric symptoms [28]. No difference was found between the AD and VaD groups. These effects have been confirmed by several meta-analyses, indicating that ginkgo treatment stabilises or slows decline in cognition, function, and behaviour [26, 29, 30]. A recently published systematic review, in which nine relatively high quality clinical trials (six studies included participants with VaD or mixed dementia) were recruited, reported that EGb761 not only enhances scores of neurocognition but also improves activities of daily living in patients with AD and/or VaD/mixed dementia [30].

3.2. Huperzia serrata. *Huperzia serrata* has a long history in Chinese medicine for use in conditions including strains, swellings, schizophrenia, myasthenia gravis, and organophosphate poisoning. The key bioactive components of *Huperzia serrata* belong to the lycopodium alkaloids family including huperzine A (HupA), huperzine B (HupB), hyperzinine, carinatumin A, and carinatumin B, all of which possess antiacetylcholinesterase properties [31]. In particular, HupA became known globally after the discovery in the 1980s for its use as a potent acetylcholinesterase (AChE) inhibitor in the treatment of dementia. In addition, HupA has also been shown to exert other pharmacological effects including antioxidant, anti-inflammatory, antiapoptosis, anti-β-amyloid peptide fragmentation, inhibition of oxygen-glucose deprivation, and NMDA receptor antagonism [32, 33] (Table 2).

The majority of clinical trials of HupA to date have been conducted in China and have investigated its effect in AD patients. A meta-analysis of HupA for the treatment of AD identified 11 studies (one open-label study, two case reports, and eight controlled clinical trials), among which 4 trials involving 474 patients (235 in the HupA treatment group and 239 in the control group) were included in the final analysis [34]. The results demonstrate that HupA (300–500 μg/day) significantly improves cognitive function, as assessed by the mini-mental state examination (MMSE) and activities of daily living (ADLs). Similarly, a recent Cochrane systematic review of six clinical trials with a total of 454 patients concluded that HupA treatment led to an improvement in general cognitive function, global clinical status, behavioral disturbance, and functional performance, with minimal side effects in AD patients [35]. Another systematic review and meta-analysis of 20 RCTs involving 1823 participants showed beneficial effects of HupA (200–800 μg/day) on improvement of cognitive function, as measured by MMSE, Hasegawa Dementia Scale (HDS), and ADL [36], although the authors note that findings should be interpreted with caution due to the poor methodological quality of the included trials.

Table 1: Nomenclature, key bioactive compounds, and mechanisms of action of commonly used herbs for VaD.

Botanic name	Chinese pinyin or other names	Key bioactive compounds	Possible mechanisms of action associated with VaD
Ginkgo biloba L	Yin Shing Ye, Ginkgo leaf, EGb761 (standardized ginkgo extract)	Quercetin, kaempferol, ginkgolides (e.g., ginkgolide B, ginkgolide C), and bilobalide	(i) Antioxidant via decreasing oxygen radical discharge (ii) Anti-inflammation via decreasing proinflammatory functions of macrophages (iii) Increase glucose uptake and utilization and ATP production (iv) Improve blood flow by increasing red blood cell deformability and decreasing red cell aggregation (v) Induce nitric oxide production (vi) Inhibit platelet activating factors
Huperzia serrata	She Zu Shi Shan, toothed clubmoss, huperzine A (key bioactive extract)	Huperzine A (HupA), huperzine B (HupB), hyperzinine, carinatumin A and B	(i) Potent antiacetylcholinesterase (ii) Antioxidant (iii) Antiapoptosis (iv) Anti-β-amyloid peptide fragment (v) Inhibition of oxygen-glucose deprivation (vi) MMDA receptor antagonism
Curcuma longa	Jiang Huang, turmeric, ginger yellow	Curcumin, demethoxycurcumin, bisdemethoxycurcumin, curcuminoids, turmerone, zingiberene	(i) Antioxidant *via* inhibiting lipid peroxidation, scavenging ROS, and reactive nitrogen species (ii) Anti-inflammation (iii) Block aggregation and fibril formation (iv) Cholesterol-lowering properties
Ginseng *(Panax ginseng, Panax notoginseng)*	Panax ginseng: Ren Shen, ginseng, Korean ginseng *Panax notoginseng*: San Qi, Sanchi, Chinese notoginseng	Ginsenosides Rb1, Rg1, Rg2, Rg3, Rg5, Rc, Rd, Re notoginsenosides R1, R2, R3	(i) Antioxidant (ii) Antiapoptosis (iii) Anti-inflammation (iv) Reduce amyloid-β and cholinesterase activity (v) Decrease blood pressure and enhance blood perfusion.
Bacopa monnieri	Brahmi	Bacosides (e.g., bacoside A, bacoside B, etc.) brahmine, nicotine, herpestine	(i) Antioxidant activity (ii) Free radical scavenging (iii) Increased cerebral blood flow via vasodilation (iv) Restoration of synaptic activity and improving nerve impulse transmission (v) Modifying ACh level (vi) Binding and detoxification of metal ions (metal chelation) (vii) Removal of β-amyloid deposits (viii) Antidepressant
Crocus sativus	Xi Hong Hua saffron	Crocin, crocetin	(i) Antioxidant effect (ii) Antiapoptosis (iii) Anti-inflammation (iv) Antidepressant (v) Antiplatelet aggregation
Camellia sinensis	Cha green tea	Polyphenols (e.g., epigallocatechin-3-gallate), caffeine, amino acids	(i) Antioxidant effect (ii) Antiapoptosis (iii) Anti-inflammation (iv) Elevation of α-secretase activity and inhibition of β-secretase activity

Table 2: Multitarget mechanisms underlying pharmacological effects of SLT components.

Therapeutic targets associated with VaD	Ginseng				Ginkgo		Saffron		
	Rg1	Re	Rb1	Rd	Ginkgo flavonoids	Ginkgolides	Total flavone-glycosides	Crocetin	Crocin
Excitatory amino acid			X	X	X	X	X		
Energy depletion						X	X		
Calcium overload			X	X		X	X		X
Inflammation cascade				X		X			
Oxidative stress	X	X				X	X		
Cholinergic system	X	X					X		
Apoptosis	X		X				X		
Cytoskeleton						X	X		
Antithrombus	X						X		
Fibrinolysis	X						X		
Platelet aggregation					X	X	X	X	X
Cerebral circulation	X				X		X		

Among studies which have included VaD patients, one Cochrane review investigated HupA for VaD but only identified one small study involving 14 participants in which HupA was found to be no better than placebo [37]. A subsequent meta-analysis of placebo-controlled RCTs of HupA on patients with AD and VaD identified eight AD and two VaD trials with 733 and 92 participants, respectively [38]. HupA treatment (100–500 μg/day) was shown to significantly improve the MMSE and ADL scores of AD and VaD patients, and longer treatment duration resulted in improved efficacy for AD patients. However, as noted in these reviews, the lack of quality data, small sample sizes of individual clinical trials, and short intervention periods limit firm conclusions about HupA's clinical efficacy, highlighting the need for rigorous randomised controlled trials with large sample sizes.

3.3. Curcuma longa. *Curcuma longa* (turmeric) is a food spice and colouring agent used in Chinese, Hindu, and Ayurvedic medicine for centuries has been applied in therapeutic preparations to treat numerous conditions such as pancreatitis, arthritis, cancer, and inflammatory, neurodegenerative, and digestive disorders. Curcumin and curcuminoids are the key bioactive components of turmeric consisting of three structurally closely related chemical components: curcumin, demethoxycurcumin, and bisdemethoxycurcumin [39]. Data from animal and/or *in vitro* studies suggests that curcumin can affect multiple pathological targets associated with dementia via inhibiting lipid peroxidation, scavenging reactive oxygen species (ROS), and reactive nitrogen species, inhibiting NF-kB activation, and its anti-inflammatory actions [31, 40]. It has also been suggested that curcumin is able to directly bind small beta-amyloid species to block aggregation and fibril formation [41].

Animal studies have shown that curcumin offers protective effects against VaD by exerting antioxidant and anti-inflammatory effects. A lower prevalence of AD in some Asian populations has been attributed to a curcumin-rich diet. One population-based study of 1,010 Asian seniors without dementia showed that consumption of turmeric containing curry was associated with improved cognitive function as measured by MMSE [42]. Based on these findings several clinical trials have been initiated [43–45]. One pilot randomised, double blind, placebo-controlled trial evaluated the pharmacokinetics and effects of curcumin supplementation (1–4 g/day) over six months in 34 AD patients [44]. The results of the study showed slight improvements in MMSE scores without significant side effects; however interpretation of these findings is limited due to the small sample size, short follow-up period, and lack of cognitive decline in the placebo group. In another 24 months, randomised controlled trial in 36 patients with mild-moderate AD and 2,000 mg and 4,000 mg/day of curcumin C3 Complex® over 24 weeks failed to demonstrate any clinical or biochemical evidence of efficacy of curcumin in AD [46]. The lack of positive findings may be somewhat attributed to curcumin's relatively low solubility and bioavailability. Further studies are required focusing on the active components of curcumin to determine the therapeutic value of curcumin in the treatment of dementia.

3.4. Ginseng. *Panax ginseng* (Ren Shen) and *Panax notoginseng* (San Qi) are two important members of the ginseng species and have been used for centuries in Chinese medicine to treat atherosclerosis, hypertension, thrombosis, external injury, and pain. In addition, ginseng has shown therapeutic benefits for learning and memory and may be useful in developing supplements for the prevention or potential treatment of AD [47, 48]. The principal bioactive components of ginseng are ginsenosides (e.g., ginsenosides Rg1, Rg3, and Rg5), which have been suggested to have antioxidant, anti-inflammatory, and antiapoptotic effects [49]. In addition, ginsenoside Rg5 has been shown to reduce amyloid-β and cholinesterase activity [50], while ginsenoside Rg3 has also been shown to promote β-amyloid peptide degradation via enhancing gene expression [51–54]. In addition, research

demonstrates that *Panax ginseng* decreases blood pressure and improves blood circulation via vasodilation activities [55].

Ginseng is widely used to treat dementia-like symptoms in many Asian countries; however the majority of studies examining its effects on cognition have been studied in animals and healthy individuals. Clinical trial data suggests that ginseng modestly improves thinking and working memory in healthy volunteers. [56, 57]. Two open-label trials showed that 12-week treatment with ginseng improved AD Assessment Scale-Cognitive Subscale (ADAS-cog) scores in AD participants [58, 59].

Two recent small open-label trials demonstrate the potential therapeutic benefits of *Panax ginseng* for AD [59, 60]. In the former study, which showed significant effects on ADAS-cog and Clinical Dementia Rating (CDR) following 24-week treatment of low or high dose (4.5 g or 9 g/day) *Panax ginseng* compared to controls [58], subjects were followed up for further 2 years during which time cognitive function was evaluated every 12 weeks using the ADAS and the Korean version of the MMSE (K-MMSE). In the long-term efficacy evaluation of the effect of *Panax ginseng*, cognitive function was sustained for the follow-up period. In the latter study, in which 87 AD participants (58 in the ginseng group and 39 in the control group) were involved, 12-week treatment with *Panax ginseng* powder (4.5 g/day) produced significant improvements in ADAS-cog and MMSE scores [59]. Clinical benefits have also been demonstrated after *Panax ginseng* is combined with ginkgo in improving cognitive function in healthy subjects [14, 24, 61–63].

Less research has been conducted on *Panax notoginseng*. One randomised controlled trial in 40 people with VaD, which compared the effect of 12-week supplementation with *Panax notoginseng* to duxil, a drug that increases oxygen in brain tissue, showed that memory function significantly improved in those given the herb [64]. In a trial with 64 older adults with lacunar infarction (cerebrovascular disease), the effects of the injectable form of *Panax notoginseng* extract (Xueshuantong) were investigated. Four-week treatment of Xueshuantong significantly increased relative cerebral blood flow and improved the ADL scores, although MMSE scores showed no marked changes [65]. Large scale, long-term studies using standardized extracts are required to confirm the clinical efficacy of ginseng therapy in dementia and VaD.

3.5. Bacopa monnieri. *Bacopa monnieri* (Brahmi) has been traditionally used in Ayurvedic medicine to treat conditions including pain, asthma, fever, inflammation, and memory decline [66]. Various mechanisms may be involved in the neuroprotective and memory enhancing effects of Brahmi such as increasing antioxidant activity [67], free radical scavenging [68], binding and detoxification of metal ions [69], modifying levels of acetylcholine [68], and increasing cerebral blood flow via vasodilation [70]. The constituents responsible for improving learning and memory are attributed to steroidal saponins and bacosides A and B [71]. Bacosides enhance kinase activity and neuronal synthesis, which is linked with the restoration of synaptic activity, ultimately improving nerve impulse transmission [72].

Brahmi improves motor learning, acquisition, and retention and delays extinction of newly learned behaviour in animals [73]. A series of clinical studies have demonstrated the acute and chronic neurocognitive effects of Brahmi in healthy elderly populations [74]. A systematic review of six randomized controlled trials using a dose of 300–450 mg Brahmi per day showed that the compound preferentially enhances secondary memory, although the duration of supplementation in these trials was inadequate to substantiate the effects of Brahmi on cognition [75]. The Australian Research Council Longevity Intervention (ARCLI) study, a randomised, double-blind, placebo-controlled, 3-arm parallel-group clinical trial is currently underway in attempt to overcome this issue by examining the effect of 12-month administration of Brahmi on cognitive performance in 465 healthy participants [76]. A study to assess the effects of 6-month treatment of CDRI 08, a standardized Brahmi extract on cognitive function in AD patients, is also being planned by the same team [74].

3.6. Crocus sativus. *Crocus sativus* (Xi Hong Hua) commonly known as saffron is used in Chinese medicine as antidepressant, antispasmodic, and anticatarrhal. Data from *in vivo* and *in vitro* studies demonstrate that saffron possesses anti-inflammatory, antioxidant, and antiapoptotic properties [77]. Saffron extract has been shown to improve learning and memory function in ethanol-induced memory impairment in mice and to ameliorate cerebral ischaemia induced oxidative damage in the rat hippocampus [78, 79]. Crocin, the principal constituent of saffron and a strong antioxidant, has been suggested to be largely responsible for saffron's protective effect on the central nervous system [80]. It has also been suggested that saffron can act as an antidepressant and antiplatelet agent [81], both of which may offer additional benefits for people with dementia/VaD.

In more recent years, saffron has been used for neurological conditions [82]. A 22-week double-blind RCT in AD participants showed that saffron (30 mg/day) resulted in comparable improvements in cognition to donepezil (10 mg/day) although better tolerated [83]. Another 16-week double-blind trial in AD participants, comparing the same dose of saffron with placebo, showed saffron supplementation resulted in significantly better outcomes in cognitive function than placebo [83, 84]. These findings support the need for larger trials over a longer duration.

3.7. Camellia sinensis. *Camellia sinensis*, commonly known as tea, is widely consumed as a health beverage, especially in the form of green tea in Asia. The chief bioactive components of tea are polyphenols, caffeine, and amino acids. Catechin polyphenol constituent, epigallocatechin-3-gallate (EGCG), is the most potent bioactive component of green tea. ECCG been shown to exert neuroprotective/neurorescue activities via a wide range of mechanisms including downregulation of proapoptotic genes, elevation of α-secretase activity, and inhibition of β-secretase activity, anti-inflammation, scavenging of ROS, and stabilisation of mitochondrial function [31, 85, 86].

Animal and epidemiological studies have suggested that drinking green tea confers protection to the brain against the ageing process. An inverse correlation between tea consumption and the incidence of AD and other neurodegenerative diseases has been suggested [86], although longitudinal and cross-sectional studies investigating the effect of green tea on cognitive function have produced mixed findings.

A cross-sectional study assessing the effect of green tea consumption on cognitive function in 1,003 Japanese participants aged over 70 years showed that daily consumption of two or more cups of green tea (100 mL/cup) was associated with a lower prevalence of cognitive impairment [87]. A recent double-blind, counterbalanced, within-subjects study which compared the effect of 27.5 g green tea extract on working memory in 12 healthy subjects showed that green tea increased working memory, suggesting that green tea may be effective for the treatment of cognitive impairments in disorders such as dementia [88]. Another prospective population-based study of 723 Japanese participants with normal cognitive function at baseline found that the incidence of dementia was significantly lower in those who consumed green tea 1–6 days/week compared to those who did not consume green tea [89]. In contrast, a double-blind randomised controlled study assessing the effects of green tea consumption (2 g/day) in 33 nursing home residents with cognitive dysfunction was unable to show a significant improvement in cognitive function as assessed by MMSE [90].

In summary, multiple herbs have demonstrated potential therapeutic benefits for improving cognitive function in dementia and/or VaD. However, most of the evidence comes from preclinical research, and many of these findings have not been directly validated in people with VaD. In addition, plasma concentrations of the bioactive components of these herbs are generally below the levels able to generate meaningful pharmacological activity. One possible explanation for these clinical effects is that these bioactive components interact synergistically leading to greater pharmacological/clinical outcomes than predicted by the activity of individual components. However, the evidence to support this theory is generally lacking and further research is needed to assess synergism of herbal medicine, as detailed below in Section 4.1.

4. Complex Herbal Formulations

4.1. Mechanisms and Synergistic Effects. Multiple herbs are often combined in complex formulations in some traditional medical systems for the treatment of various diseases. Theoretically, this multicomponent and multitarget approach may be ideal for diseases that have complex aetiologies and pathophysiologies such as VaD.

In TCM, the use of multiherbal therapies in which up to 20 herbs are used underpins its unique philosophy and holistic approach. According to TCM theories, multiple herbs are included in a complex herbal formulation based on the principle of "Jun (emperor) - Chen (minister) - Zuo (assistant) - Shi (courier)." The "Jun" herb is the key therapeutic component of the formula directly targeting the disease, the "Chen" herb is included to relieve the accompanying symptoms of the disease and/or to enhance the effects of the key herb, the "Zuo" herb reduces toxicity of the herbal formula, and the "Shi" herb facilitates the delivery of active components of the formula to the target organs and/or harmonises their effects.

The Jun-Chen-Zuo-Shen theory describes a complex interactive relationship where the herbs in a complex herbal formulation interact synergistically to enhance distribution and/or ameliorate/prevent potential side effects. Some of these interactions are able to be explained pharmacologically. For example, bioactive components in herbal formulations interact improving their solubility and subsequent bioavailability, enabling them to affect multiple therapeutic targets associated with the disease and/or to enhance metabolism of toxic components thereby reducing side effects [91].

Evidence to support these beneficial interactions is very limited and results remain controversial. The paucity of data is partially caused by the lack of robust research methodologies to study the synergistic effects of multicomponent herbs or herbal formulations. The two methods most commonly used for studying synergism are the combination index (CI) and isobole method. Both methods have been developed to determine synergistic or antagonistic interactions between two or more single-entity agents acting on the same target/receptor and require the determination of a dose-response relationship of the combination and its individual components [92]. However, these methods are inadequate for evaluation of synergy in complex herbal formulations where multicomponents interact with multiple therapeutic targets/receptors [93].

The use of the systematic analysis or system-to-system (S2S) method is gaining momentum in the study of multitarget synergistic actions. Taking advantage of computational sciences, the S2S approach integrates data from literature and experimental studies. S2S analysis is conducted via a docking process during which three-dimensional structures of individual compounds of interest are matched against the known structures of relevant key therapeutic protein targets associated with the disease using computer software. However, there is general lack of information on the chemical and pharmacologic properties of bioactive components of many herbal medicines and therefore the use of the S2S method in the study of complex herbal formulations remains a challenge [92].

4.2. Clinical Evidence of Complex Herbal Formulations for VaD. The current clinical evidence to support the use of complex herbal formulations for dementia and/or VaD remains weak and controversial. There are only a limited number of reports published in English that examine the effectiveness of complex herbal formulations for VaD. For example, in a randomised, double-blind, placebo-controlled trial, the effects of a traditional Chinse herbal formulation, *Bai Wei Di Huang Wan* (consisting of 8 Chinese herbs), were examined in 33 patients with mild to severe dementia. Although the authors did not specify the types of the dementia, 91% of the patients recruited exhibited neuroimaging evidence of cerebrovascular disease and therefore it is likely

that these patients had VaD or mixed dementia. The authors found that 8-week treatment of *Bai Wei Di Huang Wang* formula significantly improved cognitive function (measured by MMSE) and ADL (measured by Barthel Index) when compared to the placebo [94]. However, the trial was not fully powered and the invention period was relatively short. Further studies with larger sample sizes and longer duration are required to confirm these results.

The effects of a seven-herb Kampo/Chinese medicine formula, *Yokukan-san* (*Yi-Gan San* in TCM) on neurocognitive function and behavioral and psychological symptoms, were investigated in an open-label trial in 13 people with VaD according to the National Institute of Neurological Disorders and Stroke-Association Internationale pour la Recherche et l'Enseignement en Neurosciences (NINDS-AIREN) diagnostic criteria. Yokukan-san consists of *Angelica acutiloba*, *Atractylodes lancea* rhizome, *Bupleurum* radix, *Poria sclerotium*, *Glycyrrhizae radix*, *Cnidium* rhizome, and *Uncarie* hook. Although 4-week treatment of *Yokukan-san* did not significantly change the MMSE scores, there was a significant change in the overall NPI (neuropsychiatric inventory) score and mean subscores for agitation and disinhibition after the treatment, suggesting potential neuropsychiatric benefits of the formula [95].

A large number of trials were conducted in China to evaluate various complex Chinese herbal formulations for the treatment of VaD. A systematic review published in 2012 detailed 47 randomised controlled clinical trials (all conducted in China) involving 3,725 people with VaD (using diverse diagnostic criteria) in an effort to assess the safety and efficacy of herbal medicines for VaD [96]. Out of 43 studies where herbal medicines were used as monotherapies, 37 reported that the herbal interventions exerted significantly greater effects than the conventional medicines (piracetam, aniracetam, hydergine, etc.) or placebos. All 4 studies in which herbal medicines were used in conjunction with conventional medicines reported better neurocognitive efficacy outcomes compared to the conventional medicines alone. However, significant methodological issues were identified in these studies including no sample size calculation, inconsistent diagnostic criteria used, differences in baseline characteristics, inappropriate randomisation, and diverse outcome measures used (some studies used instruments developed in-house). In addition, some 43 different herbal/complex herbal preparations were used in these studies. Each of these methodological shortcomings seriously impacts on the significance of these clinical findings.

A more recent meta-analysis conducted by Gong et al. [97] using strict inclusion criteria (e.g., exclusion of studies using single herbs or with short duration) included 24 randomised clinical trials comprising 2043 people with VaD (all conducted in China), although no analysis of the VaD diagnostic criteria used in these studies was provided. In a subgroup analyses, complex Chinese herbal interventions significantly enhanced cognitive function (judging by MMSE scores) when compared to piracetam (in 10 studies) or placebos (in 3 studies). No difference in MMSE scores was identified between the Chinese herbal medicines and hydergine in 17 of the studies. Yet, herbal medicine treatments produced a greater improvement in ADLs compared to piracetam treatment in 5 of the studies. Having said this, similar methodological problems to the previous systematic reviews were identified in these studies.

In summary, a number of complex herbal formulations have been trialled clinically but mainly in China. There are numerous methodological problems with most of these clinical trials. In addition, no studies reported the standardization of their herbal interventions used in the trials. Mechanistic studies to evaluate mechanisms of action and synergy of these formulations are also lacking.

5. SLT, a Case Study on the Development of a Complex Herbal Formulation for VaD

Recognising the lack of therapeutic options for the treatment of VaD, a combined team from the China Academy of Chinese Medical Sciences and Western Sydney University (authors of this article have contributed to the project in various ways) has been working together to develop a standardized complex herbal formulation, SLT (Sailuotong, previously known as WNK) for the treatment of VaD. SLT represents a new generation of herbal medicine whereby the chemical and pharmacological profiles have been clearly defined. Over the last 10 years, a substantial body of evidence has accumulated in support of the use of SLT for VaD. Some of the key data have been summarised in this section by way of providing an example to demonstrate the developmental process of new complex herbal interventions for the treatment of serious diseases such as VaD.

5.1. SLT Development Process. SLT formula consists of specific dosages of standardized *Ginkgo biloba* (ginkgo), *Panax ginseng* (ginseng), and *Crocus sativus* (saffron) extracts, designed for the treatment of VaD. The herbs were selected based on their traditional use and existing clinical and pharmacological evidence (as summarised in Sections 3.1, 3.4, and 3.6). A number of bioactive components have been identified in ginseng, ginkgo, and saffron that have a variety of pharmacological effects associated with VaD. The effects of these key bioactive components of SLT formula are summarised in Table 2.

In the development of SLT, bioassay-guided fractionations were used to determine optimal organic solvent-based extraction methods for each herb individually. The optimal dose ratio and dosage regimen were determined through a series of pharmacological studies using various animal models. These models include VaD/ischemia models (e.g., bilateral common carotid artery occlusion model in rats, MCAO-induced cerebral infarction in rats), acquired memory impairment models induced by D-galactose, scopolamine, reserpine, and alcohol, and platelets aggression model in rabbits. The development process of SLT is summarised in Figure 2.

Based on these studies, a new formulation was developed and standardized through a rigorous quality control program where 10 bioactive markers were quantified in the final product. The pharmacodynamic and pharmacokinetic

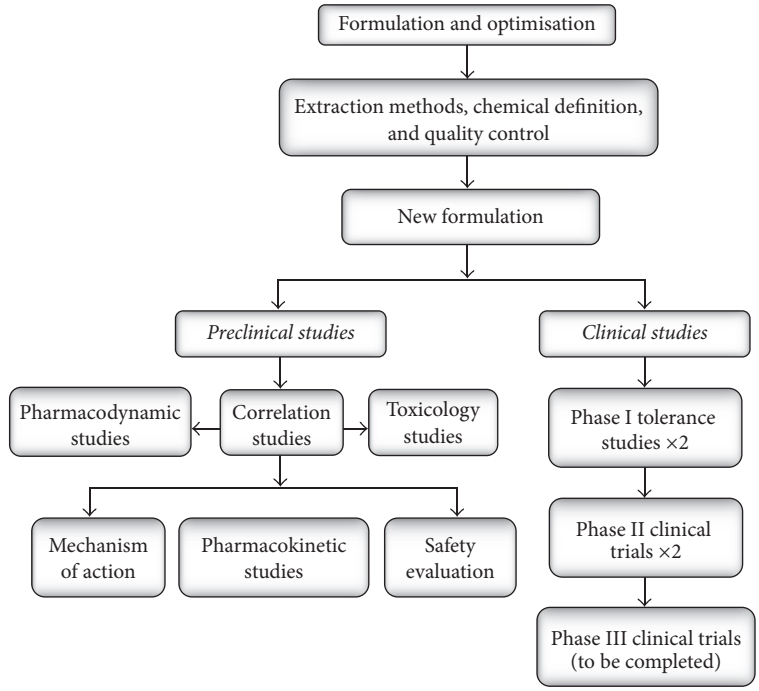

Figure 2: Development process of SLT, a novel, standardized complex herbal formulation for VaD.

properties of the formula were determined and the acute and chronic toxicity evaluated in animals. Human tolerability of the formula was determined in single administration and multiple (continuously) administration phase I studies and the clinical effectiveness, optimal clinical dose, and safety were determined in phase II studies. The efficacy and safety will be further evaluated in phase III studies where a greater number of VaD participants will be recruited.

5.2. Pharmacological Investigations of SLT. A series of preclinical pharmacokinetic and pharmacodynamic studies were conducted on SLT formula and its individual components. The data from these experiments demonstrated significant improvements in learning and memory function, pathogenic biochemical parameters in blood and brain tissue, and antioxidant capacity in various experimental dementia models.

In an *in vivo* study, the effect of SLT treatment (11, 22, and 44 mg/kg per day over 15 days) on memory impairment was investigated in acquired/consolidated dysmnesia models in mice induced by scopolamine, reserpine, chlorderazin, sodium nitrite, and alcohol. Compared with the control group, the medium and high dose treatments of SLT markedly decreased the error numbers and prolonged the latencies in all dysmnesia groups receiving active treatments, suggesting that SLT possesses beneficial protective effects on chemically induced cognitive impairments [98].

In a chronic cerebral hypoperfusion model induced by bilateral common carotid artery ligation in rats, 8-week treatment of SLT intragastrically (ig) significantly shortened the persistent time of finding the platform in a Morris water maze task [99]. Activity of cholinesterase was also significantly decreased ($p < 0.05$) while the acetylcholine (ACh) level was markedly increased in the brain tissue ($p < 0.05$). In addition, the activity of superoxide dismutase (SOD) was significantly enhanced ($p < 0.05$) [99]. These results suggest that SLT treatment improves hypoperfusion-induced cognitive impairments and this change may be associated with the cholinergic protective effect and free radical scavenging capacity of SLT formula.

The effects of SLT on ACh were also investigated in an amyloid β-protein induced dementia model in mice [52]. One-month treatment of SLT (15.5 mg/kg and 31.0 mg/kg per day, ig) significantly increased the ACh levels in the brain tissue by 18.6% and 20.0%, respectively, when compared with the model group. In another study, a longer treatment of SLT over 12 weeks at 31 and 62 mg/kg per day ig significantly increased the ACh levels in hippocampus in both treatment groups in a PDAPP^{v7171} transgenic dementia model in mice. In addition, hippocampal serotonin level was decreased significantly in the high dose SLT group [100].

The effects of crocin alone (the principal active component of *Crocus sativus*) on ischemia/reperfusion injury were investigated using a global or bilateral common carotid artery occlusion model in mice [101]. Pretreatment with 20 mg/kg crocin significantly inhibited oxidative stress (MDA content; $p < 0.001$) in mice with 20 min of arterial occlusion followed by 24 hours of reperfusion. This change was accompanied by a significant elevation in total antioxidant capacity (increased SOD and glutathione peroxidase).

SLT has also demonstrated a range of cerebrovascular benefits. Acute SLT treatment over 24 h (8.25, 16.5, and

33 mg/kg) decreased the areas of focal cerebral ischaemia/reperfusion injury in rats and increased cerebral blood flow was also observed 60–180 min after administration (10 mg/kg) [102]. Seven-day SLT treatment (16.5 and 8.25 mg/kg) also showed a decrease in platelet aggregation rate and whole blood viscosity in rats [102].

In a pharmacokinetic study of SLT in rats, four bioactive components of ginkgo including bilobalide B and ginkgolides A, B, and C were found in rat plasma after oral ingestion (60 mg/kg), with a half-life between 1.6 and 2.8 h [101]. These constituents (especially bilobalide B and ginkgolide A) were also found in brain tissue indicating that they were able to penetrate through the blood brain barrier into the brain tissue.

In an *in vitro* study, the antioxidant effect of SLT was investigated in cultured human vascular endothelial cell, EAhy926. SLT (1–50 μg/mL) significantly suppressed the H_2O_2-induced cell death and abolished the H_2O_2-induced ROS generation in a concentration dependent manner comparable to gallic acid (10 μg/mL), a well-known antioxidant [103]. In addition, SLT (1–50 μg/mL) significantly suppressed H_2O_2-induced LDH release. These results demonstrate that SLT has strong antioxidative and antiapoptotic activities on vascular endothelial cells. These properties may be associated with the neurological protective effects of SLT observed in people with VaD.

5.3. Clinical Investigations of SLT. A phase 1 study of SLT was conducted in 54 participants for evaluation of tolerance and safety. In the single administration study (60–540 mg/dose, 30 participants), the following adverse events were observed: stomach discomfort, occurrence of urticaria, local skin pain, diarrhoea, itchy skin, dry mouth, heartburn, abdominal distension, dizziness, and nausea [104]. However, there was no significant difference in the proportion of these adverse events between the treatment group and the placebo group. Furthermore, the distribution of adverse events was not dose-related. In the continuous administration study, 24 healthy volunteers were randomised to receive a low (180 mg/day) and a high dose (300 mg/day) treatment of SLT or equivalent doses of placebos over 14 days. There was no significant difference in the incidence rate of adverse events between the continuous treatment group and placebo group. No abnormal SLT treatment-related changes in liver and kidney function or ECG were observed. Overall, the results of the study showed that SLT was safe and well tolerated.

Using a randomised, double-blind, placebo-controlled crossover design, the effect of 1-week of SLT treatment on neurocognitive and cardiovascular function in 16 healthy adults was assessed [105]. In comparison to placebo, treatment with SLT resulted in a trend towards improvement in neuropsychological measures of working memory (immediate recall and N-back tasks) and in the brain's electrical response when attended information is encoded in memory (nonsignificant increase in P3a amplitude and significant decrease in N1 amplitude). The study showed that short-term SLT treatment is associated with more efficient attentional processing of auditory information and increased activation of working memory processes, suggesting that SLT has the potential to improve working memory performance in healthy adults.

In a phase II randomised, double-blinded, placebo-controlled pilot clinical study, 62 patients (32 in active group, 30 in placebo group) with probable or possible VaD were recruited according to the NINDS-AIREN criteria. Patients received 16-week treatment of either active compound or identical placebo after randomisation. At completion of the treatment, mean reductions in scores of the primary efficacy parameter, ADAS-cog, were 4.18 ± 0.75 and 1.18 ± 0.58 in participants receiving SLT and placebo, respectively [106, 107]. Although there is a difference in ADAS-cog at baseline (statistically nonsignificant) between the two groups, analysis of covariance (ANCOVA) showed that the improvement was significantly greater in SLT group than those in the placebo group after controlling for baseline ADAS-cog scores. A mechanistic substudy tested brain blood flow in 18 patients (7 patients SLT; 11 patients placebo) using Single Photon Emission Computed Tomography (SPECT). It was found that SLT treatment appeared to increase blood flow to the inferior frontal and anterior temporal lobes; regions associated with memory function and auditory and speech processing. This increase was more marked on the left when compared with the baseline in the treatment group only [106, 107]. This study reported no serious adverse events.

A second phase II dose determination study of SLT (240 mg and 360 mg per day over 52 weeks) in 325 patients with probable VaD has recently been completed. The preliminary analyses of the data indicate that 12-month treatment with SLT significantly improved cognitive function. No serious adverse reactions were reported. The full study report and publication are currently under preparation.

In summary, there are a range of challenges facing the development of complex herbal formulations. These challenges include (but are not limited to) selection of appropriate herbs, development of robust extraction methods and appropriate bioassay models, control of batch-to-batch quality, consistency of final products, establishment of pharmacokinetic and pharmacodynamic properties and toxicity profiles, and evaluation of effectiveness and safety through rigorous clinical trials. The SLT project provides an excellent example to demonstrate how some of these issues can be addressed. A considerable body of preclinical and clinical evidence has been constructed to support the use of SLT in VaD. Further work is now underway to further validate the effectiveness and safety of SLT with a greater VaD population in two multicentre phase III clinical trials. The preliminary trials detailed above provide promising findings that if supported by the phase III studies may lead to a breakthrough treatment for VaD.

6. Summary and Conclusion

Preliminary evidence demonstrates the potential therapeutic benefits of herbal medicine either as single preparations or as complex herbal formulations for the treatment of dementia, VaD, or mixed dementia. However, much of the evidence comes from animal and *in vitro* studies and overall clinical

evidence to support these herbal interventions especially complex herbal preparations remains weak.

Multiple methodological issues have been identified in existing clinical trials. In addition to the general problems associated with sample size calculation, randomisation process, and statistical analysis of the results, several dementia/VaD trial specific issues also exist. For example, many trials did not specify the diagnostic criteria used to define AD, VaD, and/or mixed dementia while, in other trials, diverse diagnostic criteria such as DSM-III, DSM-III-R, DSM-IV, ICD-10, ADDTC, HIS, and NINDS-AIREN were used. There is also a lack of consistency in the instruments used for assessment of cognitive function and some instruments that were used have not been validated in dementia and/or VaD cohorts.

The treatment durations in the existing trials are also of concern. Dementia is a progressive disease and therefore longer duration clinical trials are required to appropriately assess the effects of interventions on the disease progress. The guidelines on medical products for AD and other dementias published by the European Medicines Agency mandate that controlled clinical trials aiming at demonstrating short-term improvement in AD and VaD should last at least 6 months and 12 months, respectively [108]. In addition to cognitive functions, the changes in ADL, global clinical improvement, quality of life, neuropsychiatric symptoms, and carer burden should also ideally be investigated in the future trials.

Few mechanistic studies exist to assess the synergistic effects among the multiple herbs and/or multiple active components. This is by and large caused by the lack of robust methods to evaluate synergy. As such, more research is urgently needed in this area. Herbal medicine, particularly Chinese herbal medicine, has been widely used for the control of various risk factors such as hypertension, atherosclerosis, and diabetes associated with cardiovascular disease and VaD. However, no studies were identified that assessed the prevention of VaD using herbal medicines. A number of studies evaluating the conversion of mild cognitive impairment to AD using long-term ginkgo treatment failed to demonstrate positive outcomes [109, 110]. Future epidemiological and clinical studies are required to further assess the benefits of herbal medicines for the prevention of dementia and/or VaD.

In conclusion, the existing evidence to support the use of single and complex herbal preparations is promising but requires further development. There are numerous issues relating to the trial design in clinical studies of herbal medicines for dementia and VaD. The case study outlined here demonstrates the feasibility and potential of developing evidence-based herbal medicines for the treatment of VaD.

Competing Interests

The authors have received funding support from universities, government agencies, and industry in China and Australia in the development of SLT formula (as discussed in Section 5). The funding bodies include Ministry of Science and Technology of China, National Natural Science Foundation of China, Academy of Chinese Medical Sciences of China, Shineway Pharmaceutical Group, National Health and Medical Research Council of Australia, and Western Sydney University.

Authors' Contributions

Dennis Chang and Jianxun Liu contributed equally to this work. All other authors have made significant contributions in the preparation of this manuscript.

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Review Article

The Roles of Exercise and Yoga in Ameliorating Depression as a Risk Factor for Cognitive Decline

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Currently, there are no effective pharmaceutical treatments to reduce cognitive decline or prevent dementia. At the same time, the global population is aging, and rates of dementia and mild cognitive impairment (MCI) are on the rise. As such, there is an increasing interest in complementary and alternative interventions to treat or reduce the risk of cognitive decline. Depression is one potentially modifiable risk factor for cognitive decline and dementia. Notably, exercise and yoga are two interventions known to both reduce symptoms of depression and improve cognitive function. The current review discusses the efficacy of exercise and yoga to ameliorate depression and thereby reduce the risk of cognitive decline and potentially prevent dementia. Potential mechanisms of change, treatment implications, and future directions are discussed.

1. Introduction

Globally, the population is aging, and disorders associated with cognitive decline, including dementia and mild cognitive impairment (MCI), are on the rise. Research is increasingly aiming to identify modifiable risk factors for cognitive impairment. Psychological disorders such as mood and anxiety disorders are highly prevalent in older populations [1]. These disorders are associated with cognitive deficits, including poor concentration, attention, and memory [2–4]. At the same time, there is growing evidence to suggest that mood and anxiety disorders may be risk factors for cognitive decline in later life [5]. Indeed, in older adults, dementia is associated with similar, overlapping symptoms to depression and delirium, and careful differential diagnosis is required to accurately confirm that [6, 7]. In this way, amelioration of low mood and depression may assist in improving symptoms of poor cognition in individuals either with or without a diagnosis of dementia or MCI. Beyond this though, these findings suggest that treatment and prevention of mood disorders prior to the onset of older age and/or cognitive decline may prevent or at least slow the development of clinically significant deficits in cognitive function.

Recent systematic reviews and meta-analyses have confirmed that both mid- and late-life depression are associated with an increased risk of dementia [8–10]. Indeed, the risk of Alzheimer's disease (AD) is almost doubled, and the risk of vascular dementia (VaD) is almost tripled, if an individual presents with clinical symptoms of depression in mid- to late-life [8–10]. Due to the high rates of cooccurrence between depression and dementia there is also the suggestion that depression may be part of the dementia prodrome. Interestingly, emerging evidence suggests that first presentation of depression later in life may reflect prodromal AD, whereas chronic or recurrent life-time depression may be associated with long-term cerebrovascular changes that predispose an individual towards the development of dementia, including VaD [8, 11] and AD [10, 12]. Furthermore, the presence of

depression in individuals showing signs of cognitive decline (i.e., MCI) increases the risk of developing AD [13].

Depression is therefore a potentially modifiable risk factor for cognitive decline and dementia [14, 15]. Current first-line treatments (pharmaceutical and psychological interventions) demonstrate moderate success; however, nonresponse occurs in up to 50% of cases and many individuals fail to maintain long-term improvements [16–19]. Similarly, there is no cure for dementia and no treatment to reverse cognitive decline [20]. At best, some treatments help to alleviate certain cognitive or behavioral symptoms [21]. This highlights the need to investigate complementary and alternative treatments. Alternative therapies are often appealing to individuals who have found little or no improvement through standard interventions. Advantages of these interventions include reduced cost [22] (allowing greater access to at-risk populations and potentially producing a more sustainable long-term intervention), fewer side-effects, high levels of acceptability, and high congruence with popular culture (potentially increasing compliance). Beyond this, complementary and alternative interventions may have the potential to prevent future symptoms, in a way that is not evident with pharmaceutical interventions. In this review, we discuss the potential for two complementary and alternative interventions, exercise and yoga, to target depression and therefore reduce a significant risk factor for cognitive decline and dementia.

2. Exercise to Treat Depression

Both physical activity and exercise (i.e., a subset of physical activity that is planned, structured, and repetitive and has physical fitness as a final or an intermediate objective [23]) have been identified as having prevention and treatment effects on depression [24–26]. Moderate intensity, supervised exercise programs of at least nine-week duration (trials typically range from 8 to 16 weeks), utilising a combination of aerobic and resistance based exercise sessions, and a group format appear to have the greatest impact on reducing depressive symptoms [27, 28]. The largest community-based trial of exercise for major depressive disorder (MDD) involved 946 outpatients randomized to either 12 weeks of prescribed exercise, Internet-based cognitive behavioral therapy (CBT), or usual care consisting of brief CBT-focused therapy and antidepressant treatment and found that supervised exercise three times per week resulted in significantly lower depression severity compared to usual care and was equivalent to the Internet-CBT group [29]. These results are promising from an implementation perspective, especially for those groups for whom significant barriers to accessing standard clinician-delivered CBT exist, including stigma and financial costs associated with seeking treatment.

Nonetheless, there is ongoing debate within the scientific literature regarding the magnitude of effect of exercise on depression. Methodological differences between recent systematic reviews and meta-analyses [30], specifically around (i) the argument for and against the inclusion of pragmatic interventions such as physical activity counseling and yoga, which many argue better reflect real world clinical practice [31], and (ii) a large control group response (SMD −0.9) across exercise and depression RCTs [32], have resulted in an underreporting of the overall effect size. A 2016 meta-analysis of 25 RCTs including people with a diagnosis of depression and those with depressive symptoms found a large and significant overall effect on depression after adjustment for publication bias with an SMD of −1.11, corresponding to an approximate 5-point reduction in the Hamilton Rating Scale for Depression (HAM-D) and a greater than 6-point reduction in the Beck Depression Inventory (BDI), both of which are considered clinically significant based on the National Institute for Health and Care Excellence (NICE) guidelines [24, 33]. Additionally, it was found that a total of 1,057 negative studies would be required to nullify the significance of the main analysis, further justifying the robustness of the analysis [24]. These data are consistent with a 2015 meta-analysis of 23 RCTs investigating the effect of exercise on depressive symptoms of adults with neurologic disorders including AD which found a small but significant overall effect size of 0.28, with stronger effects of interventions meeting recommended physical activity guidelines [34]. In a subsequent 2016 meta-analysis, Schuch et al. reviewed the literature relating to exercise for depression in older adults (60 years) and found a large, significant effect of exercise on depression across the eight included RCTs (SMD = −0.90) [27].

In addition to large treatment effects, the preventative role of physical activity is becoming increasingly clear. A 2013 review of 25 studies found that baseline physical activity was negatively associated with a risk of future depression, with evidence for an effect with even low levels of physical activity (i.e., less than the recommended weekly total of 150 minutes [35]) [25]. Evidence for the bidirectional relationship between physical activity and depression continues to increase, with a recent longitudinal study of more than 1,000 participants showing that positive lifestyle behaviors (e.g., physical activity) at baseline were associated with a 22% (RR 0.76) reduced risk of episodes of a mood disorder at five-year follow-up [36]. These data are in line with a 2014 meta-analysis of observational studies finding that sedentary behavior, independent of physical activity, is associated with a significantly increased risk of depression [37]. This then becomes a vicious cycle, as people experiencing depression engage in significantly less physical activity compared to the general population [38, 39].

3. Exercise to Improve Cognitive Function

Exercise is increasingly acknowledged as having a positive effect on cognition in both the general and clinical populations, including those with depression. Evidence suggests exercise has modest effects on aspects of cognitive performance, including attention, processing speed, memory, and executive functioning [40]. While there is emerging evidence of a dose-response relationship between exercise and cognition [41], even low doses of exercise appear to provide attention and visuospatial benefits for both healthy and

depressed older adults [42, 43]. For example, improvements in attention and inhibitory control but not working memory have been found following a single, moderate intensity (65–75% of estimated maximal heart rate) exercise session [43]. Subsequent analysis of data from a large study of exercise for depression found that individuals with MDD who received a public health guideline concordant dosage of exercise (equivalent to 30 min of moderate intensity aerobic exercise, five or more days per week) had improved psychomotor speed, attention, visual memory, and spatial planning at 12-week follow-up [41].

In addition to aerobic exercise there is promising and increasing evidence for the role of strength (resistance) training in promoting cognition among older adults. In arguably one of the most comprehensive studies to date, Suo et al. found that, among 100 older individuals (mean age 70.1 years) with MCI, six months of progressive resistance training but not computerised cognitive training significantly improved global cognition [44]. Interestingly, the computerised cognitive training program but not the exercise intervention attenuated decline in overall memory performance. The available evidence suggests that targeted exercise programs are feasible, acceptable, and effective in improving aspects of cognition among vulnerable populations including older adults.

4. Yoga to Treat Depression

Yoga is an integrative mind-body practice that combines physical activity (postures or asanas) with mindfulness practices (breath control (pranayama) and meditation (dhyana)), typically performed concurrently. Several recent systematic reviews and meta-analyses have demonstrated that yoga is effective in ameliorating symptoms of depression across a range of different clinical disorders, including MDD [45–48], posttraumatic stress disorder (PTSD) [49], stress and anxiety [50], and schizophrenia [51], as well as at-risk samples, such as pregnant women [52, 53], and individuals with chronic illnesses (multiple sclerosis [54], cancer [55], and fibromyalgia [56, 57]). Yoga also improves sleep [58], a known transdiagnostic and risk factor for psychopathology [59, 60]. Importantly, one study demonstrated significant improvements in depressed mood, well-being, and self-efficacy for a group of older adults (65–92 years), compared to either a control or exercise group [61]. Similarly, yoga improved mental/emotional wellness in a small group of older adults (66 years) [62]. These studies suggest that yoga may have beneficial effects at any age, though longitudinal studies are needed to determine whether there is a crucial period for prevention of cognitive decline.

A major confound within the literature is the heterogeneity in methodology, including type of yoga intervention (particularly in the ratio of asana versus pranayama), control group, and duration and intensity of the intervention. In particular, yoga treatment studies do not always offer dosage equivalency to other standard treatments for depression [45]. While yoga appears to demonstrate a significant advantage in comparison to a no treatment or passive control group, findings are less conclusive when compared to active controls. For example, one study found yoga to be less effective than electroconvulsive therapy (ECT) but at least equivalent to antidepressants [63]. In contrast, a recent systematic review and meta-analysis found in favour of yoga when compared to aerobic exercise or usual care (including group therapy, support group, or pharmaceutical intervention, but also wait-list control) [47]. While some studies suggest a cumulative psychological advantage in long-term meditators [64, 65] and lower rate of depression remission at 9-month follow-up for group yoga [66], overall, there is a paucity of longitudinal follow-up studies investigating the long-term effects of yoga.

5. Yoga to Improve Cognitive Function

A recent meta-analysis concluded that yoga is associated with overall moderate improvements in cognitive function, particularly attention, processing speed, executive function, and memory [67]. Acute intervention studies demonstrated more consistent cognitive improvements and stronger effects sizes than RCTs (g = .56 versus g = .33, resp.). Similarly, a recent systematic review confirmed that yoga improves executive function in both healthy individuals and those with chronic illnesses such as multiple sclerosis or type 2 diabetes mellitus [68]. A cross-sectional study of older adults (>55 years) classified as long-term yoga practitioners (>10 years) demonstrated significantly superior performance on multiple tests of attention than an age- and education-matched comparison group [69]. Importantly, yoga significantly improved cognitive impairments *and* depressed mood in a group of individuals with MDD [70], demonstrating scope for treatment across multiple domains. However, as with the literature regarding yoga interventions for mood difficulties, major limitations exist in the literature for yoga as a cognitive enhancing intervention. Very few studies provide sufficient information regarding the yoga intervention and the proportion of time spent practicing each of the major elements (i.e., asanas, pranayama, and dhyana) [67]. At the same time, yoga is inherently a mind-body exercise, and arguably, it is difficult to completely disentangle the various components (i.e., part of performing an asana correctly is to also simultaneously and concurrently employ pranayama and dhyana). Nonetheless, there is a need to compare yoga to interventions that are not inherently mind-body, that is, physical activity/exercise interventions alone, and to mindfulness-based interventions alone. A 2010 review of 10 studies comparing yoga to physical exercise found that yoga and exercise were equally beneficial for physical health, but yoga was superior to exercise for improving cognitive function and mental health [71]. However, further research is needed, particularly on populations with clinical disorders (either mental or cognitive) and in older samples. Currently, there is little regulation or standardisation in terms of recommended dose (e.g., session duration, frequency/regularity, and long-term duration) or type (especially in terms of variations in emphasis on the major components) of yoga intervention.

6. Mechanisms of Action

Many theorists suggest that the association between depression and dementia is due to a common underlying vulnerability factor (stress), which triggers neuroinflammation and disruption of the hypothalamic-pituitary adrenal (HPA) axis [72–75]. Resulting hypercortisolism may lead to neural damage, including reduced hippocampal neurogenesis [76, 77], possibly via reductions in hippocampal brain-derived neurotrophic factor (BDNF) [78, 79]. Hypercortisolism is associated with depression [80], long-term impairments in cognitive function [81], and dementia [82]. Abnormal expression of BDNF and associated reductions in hippocampal neurogenesis are linked to depression [78, 83]. Similarly, dysfunction of hippocampal neurogenesis [84] and reduced BDNF [85] are both associated with increased risk for dementia. More recently, a 38-year follow-up study found that mid-life neuroticism is associated with an increased risk of AD [86]. This is particularly interesting given that current theories propose a common, nonspecific factor of negative affect (neuroticism) as a vulnerability factor for emotional disorders, including depression and anxiety [87, 88]. That is, either stress or neuroticism may be vulnerability factor for depression and/or dementia. However, there is also evidence for a direct link between the two; depression may trigger neuroinflammation that then sensitises the brain to other precipitants of dementia [73]. Indeed, chronic neuroinflammation creates a vicious cycle that may maintain depression and increase the chances of permanent long-term damage [74], including cognitive decline and dementia. While further research is necessary to clarify cause and effect relationships, particularly the extent to which a genetic predisposition is necessary, breaking the inflammatory cycle and/or hippocampal neurogenesis is likely to be beneficial for preventing both depression and dementia.

Both acute and chronic responses to exercise have been identified, including increases in atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), copeptin, and growth hormone, as well as chronic adaptations of copeptin and thiobarbituric acid reactive species (TBARS) [89]. In a recent review of the neurobiological effects of exercise on depression, Schuch et al. [89] reported that the mechanisms by which exercise affects depression are yet to be clearly understood. Despite limited evidence that exercise promotes neurogenesis and changes in inflammation biomarkers and overall brain structure, two studies included within the review reported evidence of an association between improvements in depressive symptoms and increases in hippocampus volume and IL-1B following exercise [89].

At the same time, a recent systematic review and meta-analysis confirmed that yoga has a downregulating effect on the sympathetic nervous system and the HPA axis in response to stress, including reductions in morning cortisol, blood pressure, and heart rate [48]. There is also preliminary evidence suggesting yoga may increase BDNF [90]. Mindfulness-based psychological therapies which have some overlap with yoga (e.g., mindfulness-based stress reduction (MBSR), mindfulness-based cognitive therapy (mbCT/mCBT/MBCT)) are known to downregulate the sympathetic nervous system and the HPA axis in response to stress [64, 91–94] and are associated with long-term increases in hippocampal grey matter [95]. Beyond these anti-inflammatory effects, yoga and related activities such as mindfulness meditation may improve mood and cognition via increased focus, attentional awareness, and emotion regulation and/or reduced neuroticism or negative perseverative thinking (i.e., rumination, worry) [96–100]. In this way, yoga combines both top-down (from mind to body, through mindfulness) and bottom-up (from body to mind, through reduced neuroinflammation and stress response) effects that may distinguish it from exercise, where top-down effects are a potential side-effect versus core component. Further research is needed to disentangle the unique versus shared mechanisms of action between exercise and yoga.

7. Implications for Treatment

Henceforth, the challenge ahead lies in the adaptation and translation of findings from clinical trials to person-centred initiatives, capable of delivering appropriate exercise and yoga interventions at a scalable level for people at risk of or experiencing depression and/or cognitive decline. Despite the numerous protective and treatment benefits that physical activity and yoga may offer, effectiveness of these interventions is overwhelmingly limited by the barriers to being physically active that are prevalent across society (i.e., poor motivation) and compounded among those experiencing symptoms of depression or cognitive decline [101]. While continued research into the mechanisms behind the antidepressive effects of exercise or yoga is certainly required, a focus on implementation and addressing cultural, educational, and logistical issues within treatment services is equally as important. While behavioral activation, a key component of CBT that involves scheduling activities that are pleasurable and allow opportunities for mastery [102–104], may include some form of physical activity, this review suggests that it is necessary. From a pragmatic perspective, clinical psychologists may combine this scheduling with positive data logs [105] and gratitude or savoring techniques [106–110] to increase compliance. Furthermore, programs delivered by professionals such as physiotherapists and exercise physiologists with tertiary training in exercise prescription are associated with reduced dropout and increased motivation to exercise among people with an affective disorder [111], highlighting the importance of multidisciplinary mental health teams to ensure the translation of findings into real world, scalable programs [112–114].

8. Future Directions

Important questions remain. In particular, it is currently unclear whether or not there are unique or combined effects of physical activity versus mindfulness/meditation. That is, is there something extra special about yoga (which combines the two, within the same activity), or can either individually exert similar positive benefits? Alternatively, is there an external third factor (e.g., regular routine, organised social

activity) contributing to the improvements? Currently, there is a paucity among the literature. Very few studies have compared yoga to either exercise or mindfulness/meditation as short-term interventions, for either mood or cognitive deficits. Beyond that, no studies have compared the long-term effects of any of these interventions in the prevention of either mood disorders or clinical cognitive deficits.

The heterogeneity of depression also needs to be addressed. Indeed, individuals require 5 of a possible 9 diagnostic symptoms to meet criteria for MDD, creating high internal variability amongst individuals, independent of symptom severity (see [115] for further discussion). Furthermore, do factors such as clinical symptom severity (including comorbidity) or clinical course (recurrent versus chronic, age of onset, etc.) moderate the potential benefits of exercise and/or yoga? For example, depression and anxiety are highly comorbid [116–120], which negatively affects treatment and recovery, quality of life, and global functioning, over and above the effects of either disorder independently [121–123], suggesting comorbid depression/anxiety may be quantitatively or qualitatively different than either disorder alone. Studies have also demonstrated that anxiety negatively affects cognition [2, 3, 124, 125] and that exercise [26, 46] and yoga [46, 126] may help to ameliorate these associated cognitive deficits. However, no study to date has teased apart the unique effects of exercise and/or yoga on anxiety versus depression, in relation to risk for cognitive decline. Furthermore, while it is known that age of onset of depression plays a role in the development of dementia [8, 10–13], it is unknown how this interacts with treatments. For example, is there crucial or optimal time window for intervention? Future studies should investigate the potential benefits of exercise and yoga in the context of clinical severity and course.

9. Conclusion

Depression is a potentially modifiable risk factor for dementia. Both exercise and yoga are effective treatments for depression and cognitive decline that are also relatively easy and cost-effective to implement. However, it is currently unclear which components of these interventions (e.g., physical activity, mindfulness) are necessary or sufficient to produce change. Further research is also needed to determine whether these interventions are capable of preventing or at least slowing the development of clinically significant deficits in cognitive function and, if so, the optimal timing of these interventions.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Traditional Chinese Medicine Huannao Yicong Decoction Extract Decreases Tau Hyperphosphorylation in the Brain of Alzheimer's Disease Model Rats Induced by $A\beta_{1-42}$

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Objective. Huannao Yicong Decoction (HYD, 还脑益聪方) has been shown to improve the learning and memory capabilities of Alzheimer's disease (AD) subjects. However, the underlying mechanism remains to be determined. *Methods*. Sixty Sprague-Dawley rats were divided equally and randomly into five different groups including control, positive control, and HYD granules of low dose, medium dose, and high dose by daily gavage. The sham-treated rats were also given the same volume of sterile water by gavage. Twelve SD rats were treated with the same amount of physiological saline. Twelve weeks later, learning and memory capabilities, $A\beta$ content of the right brain and the expression of glycogen synthase kinase-3β (GSK-3β), total tau protein kinase (TTBK1), and cyclin-dependent kinase-5 (CDK-5) were tested. *Results*. Our results showed that high dose HYD treatment significantly improved the learning and memory capability of the AD rats and decreased the expression of TTBK1, GSK-3β, and CDK-5 in the hippocampal CA1 region. *Conclusions*. HYD treatment for 12 weeks significantly improved spatial learning and memory and effectively inhibited $A\beta$ deposition, likely via reducing tau protein kinase expression and thus tau hyperphosphorylation and inflammatory injury. Taken together, these results suggest that HYD could be an effective treatment for AD.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease, characterized by progressive memory loss and cognitive dysfunction. Intracellular neurofibrillary tangles (NFTs), extracellular senile plaque (SP), and abnormal cholinergic transmitter metabolism in the central nervous system (CNS) are pathological features observed in AD patients upon autopsy [1]. Hyperphosphorylation of tau is believed to account for most NFTs. In the brain of AD patients, the amount of total tau protein was far greater than what is typically observed in unaffected individuals, largely driven by dramatic increases in the amount of hyperphosphorylated tau [2]. At present it is well accepted that extracellular deposition of amyloid protein ($A\beta$) may trigger AD [3]. As tau phosphorylation correlates well with AD severity and extent of cognition impairment, investigations into improved understanding of tau and in particular hyperphosphorylated tau have attracted great attention. Currently, there is no effective treatment for AD. Recent studies have highlighted a potential for HYD in improving the clinical outcomes of old patients with mild cognitive impairment (MCI) [4]. HYD is based on the mechanism of deficiency-congestion-phlegm-toxic in Chinese medicine, which has been shown to improve metabolism disorder of free radicals, reducing inflammatory immune response and regulating cholesterol metabolism [5]. Moreover, animal models have revealed the impact of HYD on cognition impairment by decreasing $A\beta$-associated proteins and inflammatory immune response and inhibiting apoptosis of hippocampal neurons to maintain normal brain function [4].

In the present study, we studied the effect of HYD on spatial learning and memory in $A\beta_{1-42}$ induced AD rats. To enhance our understanding of the mechanisms through which HYD influences AD, we also evaluated the changes in tau protein kinase expression in the hippocampus following HYD treatment.

2. Methods

2.1. Animals. All animal work described in the present study was reviewed and approved by the Committee on Ethics of Animal Experiments of Xiyuan Hospital of China Academy of Chinese Medical Sciences (Permit Number: 2011XLA10-05). A total of 72 Sprague- Dawley (SD) rats (half male and half female) at 3 months of age, weighing 200–220 g, were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd., with certificate of conformity: SCXK (Beijing) 2006-0009. The rats were raised in specific pathogen-free environment with temperature of 22–25°C, humidity of 50–70%, and a 12 h light cycle. All rats were given seven days of adaptive feeding to acclimatize.

Establishment of Animal Model and Drug Administration. Sixty rats were randomly selected for $A\beta_{1-42}$ treatment. The remaining 12 rats represented the sham group and were treated with an equivalent volume of water. Prior to experimentation, $A\beta_{1-42}$ was diluted with sterile saline to a concentration of 1 μg/μL and placed at 37°C for 72 h. For the $A\beta_{1-42}$ or sham treatment, rats were anesthetized by 4% chloral hydrate at 0.9 mL/100 g body weight and fixed on the stereotaxic instrument. The head was shaved around the fontanelle region and disinfected with 75% alcohol. A 1.5 cm longitudinal incision was made using surgical scissors. Referring to brain stereotaxic instrument map of rats by Zhuge Qichuan, the bilateral hippocampal CA1 region in the dorsal part of dentate gyrus was identified. Accurate positioning was made and marked by the stereotaxic instrument (BW-SAD902 digital explicit stereotaxic instrument, Stoelting, USA): 3 mm after anterior fontanelle, 2 mm next to the midline of the left and right brain, and 4 mm under the surface of the skull. Using a dental embossed ball drill (number 10), two holes were drilled. Five microliters of condensed $A\beta_{1-42}$ was injected slowly into each side of the hippocampal CA1 region at a speed of 1 μL/min by micro flow rate pump (TJ-2A/L0107-2A-Micro Flow Rate Syringe Pump), manufactured by Baoding Longer Precision Pump Co., Ltd. To ensure an adequate diffusion, the needle was kept in the brain for 5 min and withdrawn slowly. Yunnan Baiyao was applied to the wound to protect it from infection and bleeding, and the skin was sutured. The sham group was similarly operated on but sterile saline rather than condensed $A\beta_{1-42}$ was injected. The rats received intramuscular injections of penicillin 40000 U/day for 3 days to prevent infection and the wound was checked regularly.

The 60 AD model rats were divided into 5 groups (control, DPG, HLG, HYD-mid, and HYD-high) based on the results of initial Morris water maze test and their body weight using stratified random method with the SPSS software. The five groups received a daily gavage for 12 weeks with distilled water (water), Donepezil hydrochloride tablets suspension (0.49 mg/kg) (Donepezil), and HYD at three doses (in g crude drug/kg): 3.78 (HYD-L), 7.56 (HYD-M), and 18.90 (HYD-H), respectively. The sham group (control) received intragastric administration with distilled water. All six groups received the same total volume. During the twelve-week period, one rat from the HYD-M group died due to an accident and thus was removed from the study.

2.2. Preparation of HYD Extract and Donepezil Suspension. HYD granules, composed of *Radix Polygoni Multiflori*, *Radix Ginseng*, *Rhizoma Ligusticum wallichii*, *Rhizoma Acori Graminei*, and *Rhizoma Coptis* with a weight ratio of 2.4 : 2 : 1.8 : 1.2 : 1 were extracted with ethanol and water. The extract was concentrated under reduced pressure and then mixed with volatile oil of *Acorus tatarinowii Schott Naphtha*. The thick extract, prepared by the Drug Manufacturing Facility at Beijing University of Chinese Medicine, contained crude drug at a concentration of 3.2 g/kg. The extract was diluted with distilled water at designed ratios before being given to rats by gavage. Donepezil hydrochloride tablets (Aricept, 5 mg, National Medicine Permit: H20050978, lot number: 100609A), produced by Eisai China Inc., were crushed and mixed with distilled water as a suspension.

Other Reagents. $A\beta_{1-42}$ (lot number bs-0107b) was purchased from Sigma-Aldrich, USA. β-amyloid (β-AP) kit (batch number: 20111015) was obtained from Beijing Huaying Institute of Biotechnology. The antibodies for brain-derived tau protein kinase (TTBK1/BDTK, phosphorylated, batch number: 909902W), glucose synthase kinase-3β (GSK-3β, lot number 110267), and cyclin-dependent kinase 5 (CDK5, batch number: 110218) were obtained from Beijing Biosynthesis Biotechnology Co., Ltd. Polink-2 plus Polymer HRP Detection System (nonbiotinylated) (lot number: K116616D) and 3,3′-diaminobenzidine (DAB) chromogenic kit (lot number: K116610D) were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.

2.3. Behavior Test. Morris water maze (DMS-2 type, produced by the Institute of Materia Medica, Chinese Academy of Medical Sciences) was used before and after drug treatment to detect changes in cognitive function [6]. The test consisted of two parts: a place navigation and spatial probe test. The place navigation was evaluated daily for 4.5 days. The water in the maze was 30 cm deep and approximately 1 cm above the platform surface, which was located in fourth quadrant, with a temperature of 25 ± 1°C. During the test, an appropriate amount of ink was mixed in the water so as to turn the color opaque black. This test was conducted in a soundproof room. The procedure for this testing involved the following: (1) choosing and marking a point of pool wall from 1/2 radians of the second quadrant, (2) placing the rats against the pool wall into the water on the marked point, and (3) recording the time required to find and climb the platform (such that all limbs were on the platform). Together this procedure was called platform-locating latency (PLL). If the rats could not find the platform in 180 s, the conductor guided the rat appropriately. For the first 4 days, only PLL was recorded. On the fifth day, both PLL and swimming distance were recorded. Moreover, on the fifth day (in the afternoon), the platform was removed to perform a spatial probe test. In this test the rats were made to face the pool wall and enter the water from a random point of the second quadrant. The camera system

automatically recorded the location where the platform was located as well as the time and distance of swimming to evaluate their learning and memory ability.

2.4. Preparation of Brain Tissue Sample. After the final Morris water maze test, six rats selected randomly from each group were anesthetized and sacrificed by decapitation. The brain tissue was immediately placed on ice. The left side of the brain was fixed in 4% neutral paraformaldehyde. The other side of the brain was used to dissect out the hippocampus and cortex. Hippocampal tissue was weighed, and 0.3 g cortex was homogenized with 2 mL of ice cold buffer and centrifuged at 2000 rpm for 10 min. The supernatant was stored at −80°C for future use.

2.5. Determination of Aβ by Radioimmunoassay. Radioimmunoassay was used to determine the content of Aβ in the right brain of the rats [7]. Briefly, the β-AP antibody was added into the brain tissue homogenate, which was labeled with ^{125}I. After 2 hours, the secondary antibody was added to isolate antigen-antibody complex. The radioactivity (B) of the samples was measured together with the standards using the automatic γ-counting instrument (GC-911, China Science and Technology University of Industry Company, Ltd.).

2.6. Immunohistochemistry. The fixed brain tissue was paraffin embedded and cut into 5 μm sections. H&E and Congo red staining [8] were performed to evaluate histopathological changes in the hippocampus CA1 region of the rat brains. Five rats from each group were randomly selected for brain sections. The expression of TTBK1, GSK-3β, and CDK-5 in brain tissue was examined using a two-step nonbiotinylated immunohistochemistry, according to the manufacturer's specifications. Briefly, 5 μm brain sections were dewaxed, incubated with 3% H_2O_2 deionized water for 5–10 min to block endogenous peroxidase activity, washed 3 times in phosphate buffered saline (PBS) (2 minutes each), and then incubated with the primary antibodies (TTBK1, GSK-3β, and CDK-5; 1 : 400 dilution) at 37°C for 1 h. The slides were washed 3 times again in PBS (2 mins/wash) and incubated with rabbit anti-goat IgG secondary antibody for 30 min at room temperature. DAB chromogenic reagent was used to develop the image and the reaction time was determined by observing the tissue under microscope. The tissue was washed with distilled water, stained with hematoxylin, ethanol-dehydrated, and mounted with neutral gum. The stained sections were imaged with DpxView Pro (DeltaPix, Denmark). Three different images of the CA1 region on each section were taken, and a total of 15 images from each group were collected. The protein expression was determined by measuring the integrated optical density (IOD) with Image-Pro Plus 6.0 software.

2.7. Statistics. SPSS13.0 statistical package was used for data entry and processing. The data were expressed as mean ± SD. One-way ANOVA was used for the intergroup comparisons. LSD and Dunnett's *C* test were taken for heterogeneity of variance. A statistical significance was defined as $P < 0.05$.

3. Results

3.1. Effects of HYD on Spatial Learning and Memory Ability of Aβ-Induced AD Rats. Compared with the sham group, the AD rats had a significant decrease in the number of rats passing through the platform and the swimming time in the fourth quadrant (Figures 1(a) and 1(b)). The swimming distance of the AD rats in fourth quadrant also showed a trend to decrease although the difference was not statistically significant between the two groups (Figure 1(c)). Donepezil treatment group showed a trend of improvement (e.g., increased the number of passes and the swimming time) but such improvement was not statistically significant (Figures 1(a)–1(c)). Treatment with the low or medium dose of HYD yielded similar results as Donepezil; however, the high dose HYD treatment (18.9 g crude drug/kg) outperformed Donepezil by increasing the swimming time significantly in the fourth quadrant to that in the control rats (Figures 1(a)–1(c)). These data suggest that treatment of the AD rats with high dose HYD could improve the learning and memory abilities of these animals.

3.2. Effects of HYD on the Histopathology of Hippocampal CA1 Region in the AD Rats. In control rats, the cells in hippocampal CA1 region were lightly stained for intracellular structures and nucleus (Figure 2(a)). The cells were arranged in an organized fashion with clear boundaries. In contrast, the cells of the AD rats were stained much darker with expanded cytoplasm and darker pyknotic nuclei (Figure 2(b)). The cells were arranged in an irregular pattern as compared to the sham group with more randomly located cells. Following twelve weeks of treatment with HYD, the histopathology of the AD rats in hippocampal CA1 region showed various degrees of improvement, similar to the positive Donepezil treatment group (Figures 2(c)–2(f)). The area of cytoplasm and the number of darkly stained cells decreased as compared with untreated AD rats. The cells appeared to be more organized and the deep stained nucleus appeared lighter.

3.3. Effects of HYD on Aβ Deposition in Hippocampal CA1 Region of the AD Rats. To determine the Aβ deposition, we performed Congo red staining on the brain sections. The amount of orange amyloid deposition in the AD rats appeared to be increased as compared to sham controls and a scattered cell organization pattern was observed (Figures 3(a) and 3(b)). Following HYD treatment at three different doses or Donepezil treatment, the cells were stained lighter with clear cellular structure as compared to the untreated AD controls (Figures 3(c)–3(f)). The cytoplasm and nucleus were more clearly defined and both fewer and lighter orange amyloid deposits were observed. Consistently, the Aβ content in the hippocampus of the AD rats was significantly increased as compared with the sham group (Figure 4(a)), while all treated groups (including those treated with Donepezil and all 3 different doses of HYD) had significantly reduced Aβ content in the hippocampus (Figure 4(a)).

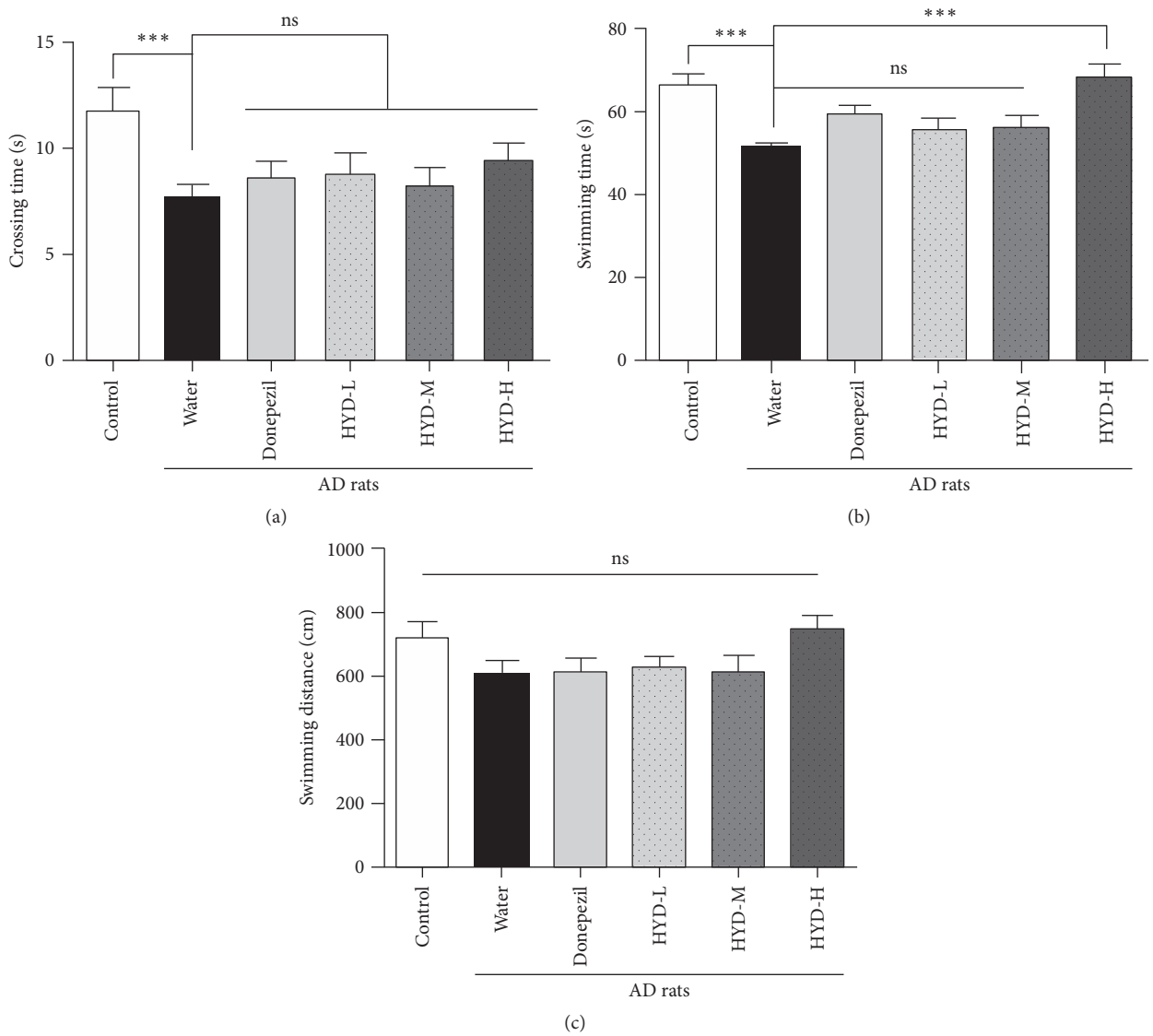

Figure 1: Effect of HYD treatment on learning and memory capability of AD rats. Control: sham treated; AD rats were fed with water, Donepezil, and HYD at low (HYD-L), medium (HYD-M), and high (HYD-H) dose, respectively, by daily gavage for 12 weeks.

3.4. Effects of HYD on the Expression of TTBK1, CDK-5, and GSK-3β in the Hippocampal CA1 Region. To test whether HYD treatment has any effect on the tau phosphorylation kinases, we performed immunohistochemistry to quantify the expression of tau phosphorylation kinases. Compared with the sham group, the expressions of TTBK1, CDK-5, and GSK- 3β staining in the brain sections of the AD rats were all significantly increased ($P < 0.001$) (Figures 4(b)–4(d)). Importantly, HYD treatment significantly decreased the expression of these proteins, similar to that in the positive control group treated with Donepezil (Figures 4(b)–4(d)).

4. Discussion

The current prevailing hypothesis of AD suggests that Aβ plays a critical role in the initiation and progression of this disease. According to amyloid cascade hypothesis, Aβ acts as a trigger in both familial and sporadic forms of AD [9]. As such, it may be effective to treat or inhibit Aβ at an early stage of the pathogenesis of AD. However, a growing number of studies have shown that once AD had been triggered, tau protein was more relevant with the severity of disease [10]. These findings have led investigators to characterize the relationship between tau protein and Aβ in AD. Moreover, it was found that Aβ depositions were cleared first and subsequently reemerged prior to the tau pathology, indicating a hierarchical and direct relationship between Aβ and tau [11].

Hyperphosphorylated tau protein is a major component of NFTs. The phosphorylation level of tau protein depends on the relative activity of protein kinases and protein phosphatases. Increased Aβ could induce tau protein phosphorylation by disrupting the balance of kinases and phosphatases. The kinases involved in the phosphorylation

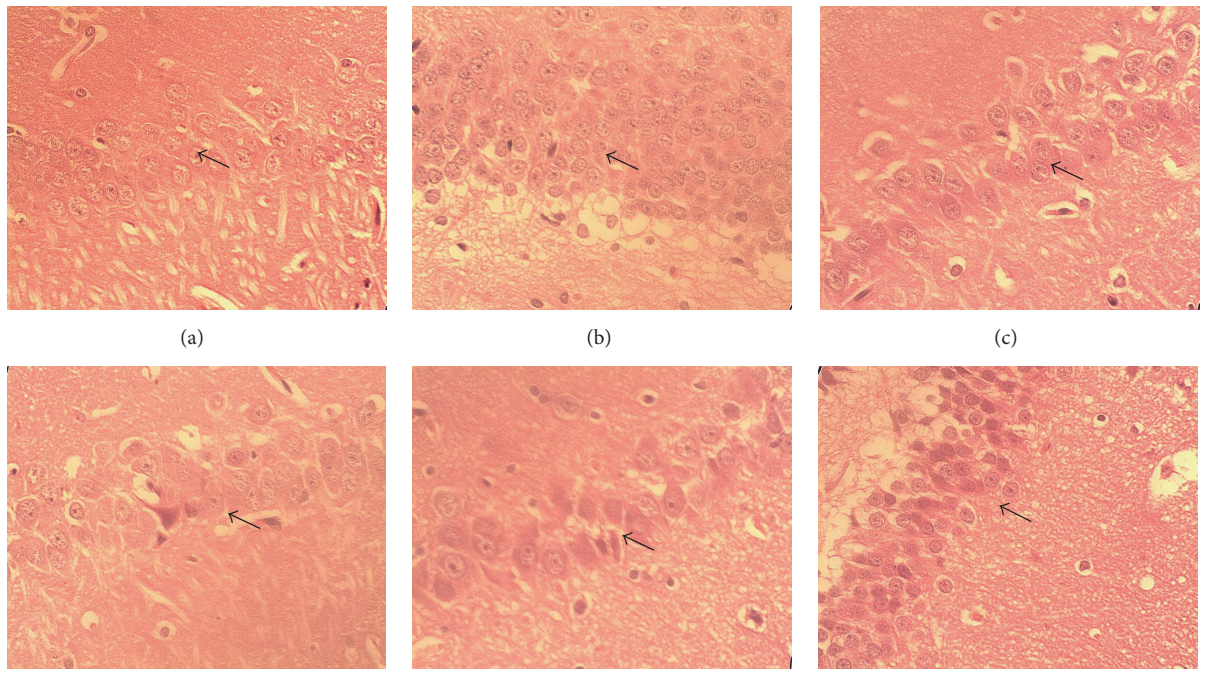

Figure 2: H&E staining (×600) of brain sections from the sham and AD rats. (a) Sham group; (b–f) AD rats fed with water (b), Donepezil (c), and HYD at low (d), medium (e), and high (f) dose, respectively, by daily gavage for 12 weeks.

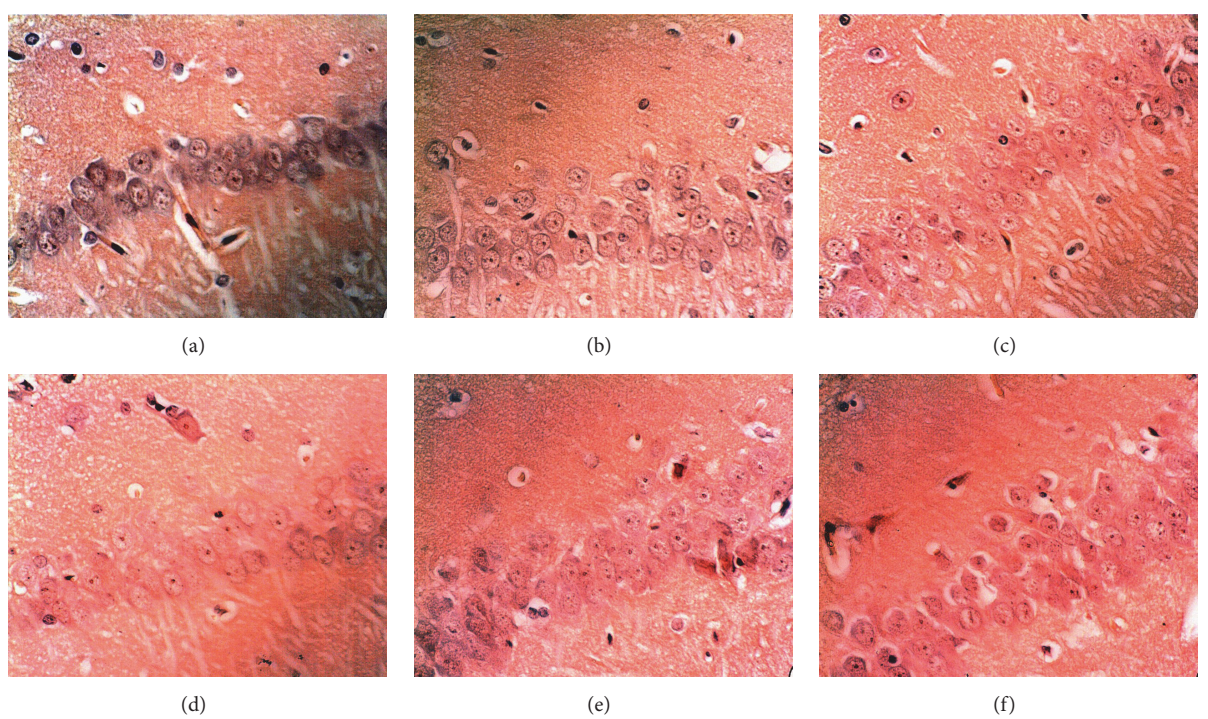

Figure 3: Effect of HYD on Aβ deposition in hippocampal CA1 region of the AD rats (Congo red staining, ×600). (a) Sham group; (b–f) AD rats fed with water (b), Donepezil (c), and HYD at low (d), medium (e), and high (f) dose, respectively, by daily gavage for 12 weeks.

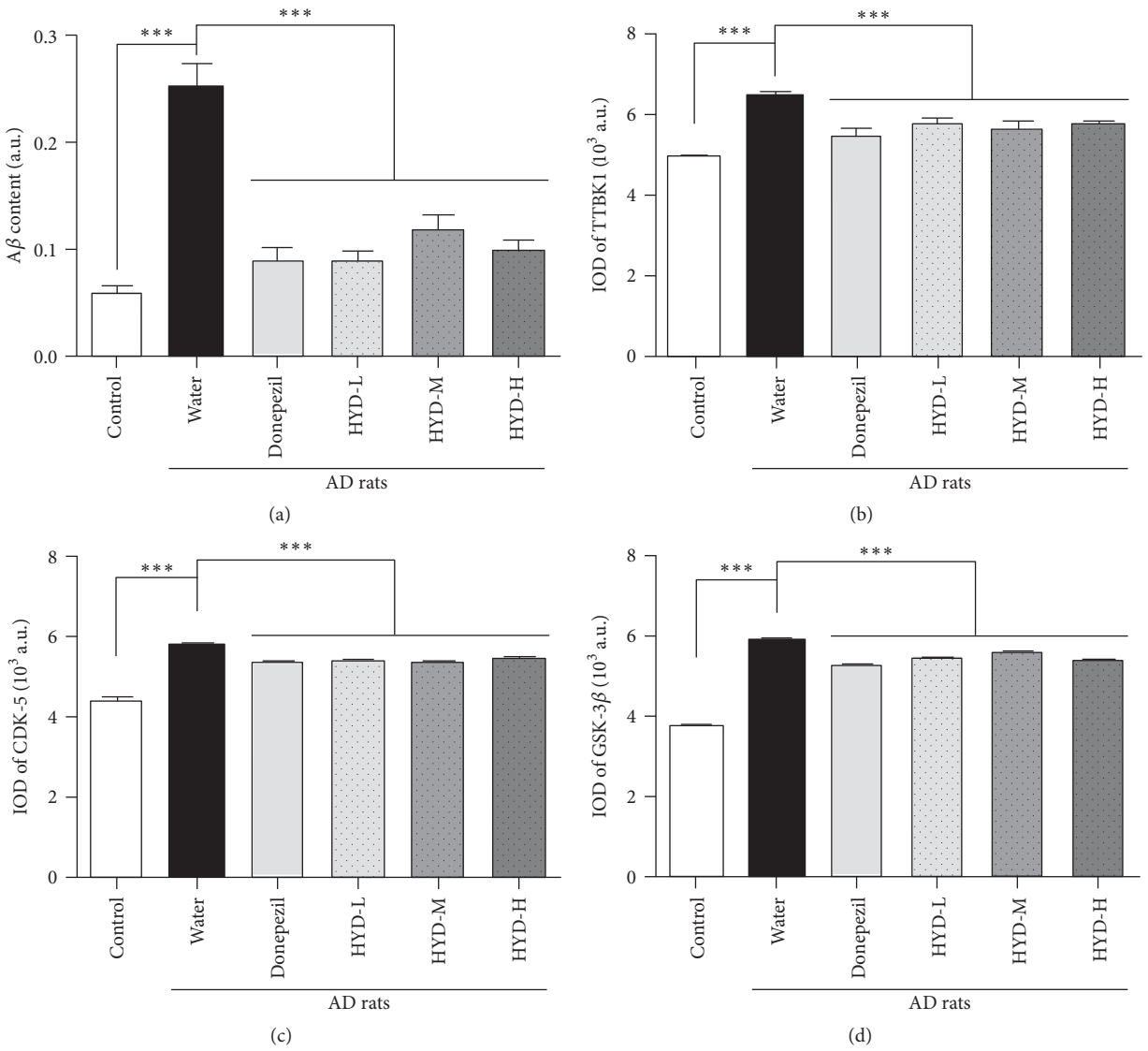

Figure 4: Effect of HYD on Aβ content and expression of TTBK1, CDK-5, and GSK-3β in hippocampal CA1 region of the AD rats.

of tau protein include GSK-3 and CDK5 [12, 13]. Interestingly, in a *Drosophila* model of AD, aberrant expression of GSK-3 and inhibition of GSK-3 both could significantly improve Aβ-induced neurotoxicity [14]. Similarly, tau defects in *Drosophila* were associated with some degree of improvement. These results indicate that GSK-3 may represent a common pathway linking Aβ and tau proteins. GSK-3 inhibition could therefore reduce the deposition of Aβ and tau protein phosphorylation [15]. Other studies have demonstrated that CDK-5 could inactivate GSK-3β and exacerbate the pathological process of NFT [16].

The AD rats demonstrated a decreased ability to find the platform in Morris water maze and, as compared with the sham group, brain tissue sections of the AD group showed obvious inflammatory lesions, increased cytoplasm to nucleus ratio, nucleus pyknosis, and an overall random cellular organization. Congo red staining identified an increased frequency of dark orange deposition of amyloid plaques in hippocampal CA1 region of model rats (i.e., Aβ deposition) as compared with the sham group. The Aβ content in the AD group also increased significantly ($P < 0.01$) as determined by radioimmunoassay. Following HYD treatment, the cognition expression in behavior was significantly improved and the severity of brain tissue pathology had been ameliorated. These results suggest that HYD repaired the cognitive impairment and neuronal pathological damage and may suppress Aβ formation and aggregation.

By immunohistochemistry, we evaluated the expression of TTBK1, GSK-3β, and CDK-5 in hippocampal CA1 regions. All three proteins were significantly increased in the AD rats as compared to the sham group ($P < 0.01$). These data suggest that Aβ affects tau protein phosphorylation, likely via inducing tau protein kinase expression. This is consistent with previous work by Tokutake et al. showing that Aβ could induce tau phosphorylation at the cellular level, and GSK-3-mediated pathway may be a gatekeeper linking these two

proteins [17]. Similarly, Chabrier et al. have shown that, by injecting APP transgenic mice with low dose of Aβ, total tau protein and tau protein phosphorylation levels increased [18]. In the present study, we demonstrate that intragastric HYD treatment significantly decreased the total amount of Aβ as well as the tau protein kinase GSK-3 and CDK-5 expression, indicating that the traditional Chinese medicine prescription HYD could reduce not only the amount of Aβ but also the expression of tau protein kinases, thereby inhibiting the phosphorylation of tau proteins. As such, the traditional Chinese medicine HYD holds a great potential for the treatment of AD.

In traditional Chinese medicine, treating dementia was based on an understanding of the mechanism of deficiency-congestion-phlegm-toxic. HYD is in line with this understanding of the pathogenesis of dementia. High performance liquid chromatography was used to determine the ingredients of the HYD extract (see Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/6840432), which was found to contain seven major active ingredients: emodin, stilbene glycoside, ginsenoside Re, ginsenoside Rb1, ginsenoside Rg1, ferulic acid, and Berberine at concentrations (in mg/mL) of 0.8045, 0.8765, 0.6932, 0.5816, 0.3993, 2.4183, and 1.0424, respectively. Recent studies have shown that emodin, one of the bioactive constituents of Radix Polygoni Multiflori, could reduce the cell death in rat cortical neurons by upregulating Bcl-2 via the estrogen receptor (ER) and phosphatidylinositol-3-kinase (PI3K) pathway [19]. Stilbene glucoside, another major extract of Radix Polygoni Multiflori, could improve the cognitive impairment by reducing oxidative stress and increasing the activity of choline acetyl transferase (ChAT). The active ingredient of Radix Ginseng Rg1 could significantly reduce phosphorylation of tau protein [20]. Li et al. established an AD model by injecting D-galactose and Aβ_{1-42} and found Rgl affects tau protein phosphorylation by inhibiting the activity of CDK5 in hippocampal neurons [21]. The active ingredient of Rhizoma Acorus could inhibit cholinesterase activity so as to delay neurodegeneration and improve cognition impairment [22]. Berberine (BR), one of the main extracts of Rhizoma Coptis, may improve the spatial memory in AD rats [23]. Durairajan found that Berberine regulates tau phosphorylation by Akt/GSK-3 and thus plays a role in protecting neurons [24]. It had been confirmed that HYD improves the ability of spatial learning and memory in various models of AD [25–27]. Although the specific mechanism of how traditional Chinese medicine affects AD model rats was not fully elucidated, our study sets the foundation for future investigation into the role of tau protein phosphorylation in the progression of AD. Collectively, the traditional Chinese medicine HYD has numerous biological effects that together may represent an important component for the development of an effective treatment regimen for patients with AD.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

All authors participated in study design, data interpretation, and preparation and critical revision of the manuscript for important intellectual content. Yu Cao, Xingxing Jia, Yun Wei, Meixia Liu, and Jiangang Liu carried out the histopathological, biochemical, and functional analyses of the rats and performed data analysis. Xingxing Jia contributed to data analysis and figure preparation. Hao Li contributed to the concepts, design, and coordination of all aspects of the experiments, interpretation of the data, and manuscript preparation and submission. All authors read and approved the final manuscript.

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Review Article

Vascular Contributions to Cognitive Impairment and Treatments with Traditional Chinese Medicine

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The prevalence of cognitive impairment and dementia caused by cerebrovascular disease is likely to increase with the global aging population. Vascular contributions to cognitive impairment and dementia (VCID) is a wide spectrum term used to include a diverse heterogeneous group of cognitive syndromes with vascular factors regardless of the cause of pathogenesis. VCID ranges from mild cognitive impairment to full-blown dementia with vascular dementia (VaD) as the most severe stage. It is further complexed by the coexistence of other forms of dementia such as Alzheimer's disease (AD). Recent researches in the functions of the neurovascular unit (NVU) suggest that dysfunction of the NVU might be the cause of primary vascular events in the brain that leads to further neurodegeneration. In this review, we have briefly summarized various forms of VCID. There is currently no standard therapy for VCID or dementia. Given the fact that Traditional Chinese Medicine (TCM) has gained popularity worldwide, we also reviewed recent scientific and clinical findings on various antidementia TCM for the treatment of VCID, including *Salvia miltiorrhiza, Huperzia serrata, Ligusticum chuanxiong, Ginkgo biloba, Panax ginseng,* and also TCM formula Sailuotong capsule (SLT) and Fufangdanshen tablets (FFDS).

1. Introduction

Dementia, which describes a syndrome with a gradual decline in cognitive functioning, is a spectrum term that includes various forms of cognitive impairment especially among the elderly of our society. In 2015, the World Health Organization (WHO) estimated 47.5 million people are living with dementia worldwide, and the number is projected to be tripled to 135.5 million by 2050 [1]. The overall prevalence of dementia among people aged 60 years and above is between 5 and 10%, varying among different global regions [2, 3]. In 2010 in China, 9.19 million of people were living with various forms of dementia, and the prevalence increases quickly with age escalating from 2.6% at age 65–69 to 60.5% at age 95–99 [4]. The most common type of dementia is Alzheimer's disease (AD), which accounts for 50–70% of all cases registered, followed by VaD, which accounts for 25% [1, 2]. Age-related dementia is a major cause of disabilities in the elderly. Apart from the financial burdens, the social stigma associated with the loss of cognitive abilities and dependency on others also causes psychological distress in patients as well as their families. The epidemic scale of dementia poses one of the biggest challenges on global public health systems and the financial burden associated with the social care needed. The pathogenesis of dementia is complex and it involves the interactions between many different physiological systems. Traditionally, AD and VaD are classified clinically as neuropathological and cerebrovascular disorders. However,

patients with AD often have mixed etiologies with both neurodegenerative and cerebrovascular pathologies [3, 5, 6]. Besides, ischemic or hemorrhagic cerebrovascular diseases or cerebral lesions caused by cardiovascular origin are commonly associated with cognitive impairments [3, 5]. Cerebral infarctions and alterations in brain blood vessels commonly occurred in the elderly which are possibly due to age-related degeneration and other diseases [7, 8]. Since the brain is a highly perfused organ and requires a continuous blood supply for its physiological functions, it is not surprising that damage to the cerebral circulation are associated with an increased risk of many types of dementia. In addition, epidemiology evidence indicates that AD and VaD share similar cardiovascular risk factors including apolipoprotein E (APOEε4), hypertension, hypercholesterolemia, obesity, and diabetes [5, 9, 10], although the strength of the association between AD and cardiovascular risk factors could be greatly influenced by the designation of parameters, and further evaluation is needed [11, 12]. Therefore, recent researches in neurodegenerative disorders such as dementia and AD have focused on understanding the interplay between vascular dysfunctions and primary neurodegenerative processes. It is also suggested that cerebrovascular pathologies may be primary causes as well as contributing factors in the progression of cognitive impairment and dementia.

In this review, we have included an overview of the definitions of various forms of dementia with vascular origins, the importance of the neurovascular unit (NVU), and the preclinical and clinical investigations of using Traditional Chinese Medicine (TCM) to treat and manage cognitive decline and dementia. Management of vascular risks and symptoms is the primary approaches in treating vascular dementia, and TCM has proven ability to treat cardiovascular diseases and hypertension effectively [3, 13]. Moreover, TCM formulations for the treatment of dementia-like and memory disorders have been extensively documented in the classical Chinese medical literature, including herbs such as *Salvia miltiorrhiza*, *Huperzia serrata, Ligusticum chuanxiong, Ginkgo biloba,* and *Panax ginseng* [14–16]. In a recent meta-analysis study, it indicated that TCM exhibited comparable efficacy and safety as Western medicine for improving the cognitive and behavior functions of patients with vascular cognitive impairment with no dementia [17]. Therefore, it is proposed that TCM has great potential uses as preventive strategies against dementia which can have positive impacts on global public health.

2. Definitions of Vascular Dementia (VaD), Vascular Cognitive Impairment (VCI), and Alzheimer's Disease (AD)

Dementia describes a group of syndromes relating to cognitive decline. Clinical manifestation of different forms of dementia exhibits different levels of impaired performance in various cognitive domains, including memory, learning, executive function, and behavioral changes (e.g., depressive symptoms). Cognitive impairment ranges from mild to severe declines in any cognitive domain. Alzheimer's disease (AD) is the most common form of dementia, followed by VaD [1, 2]. In the literature, "VaD" is often used ambiguously to describe a group of clinically similar cerebrovascular disorders associated with multiple pathological features, such as multi-infarcts, single infarcts, hemorrhages, white matter hyperintensities. Moreover, the progression of the manifestation of dementia is preceded by a prodromal stage in which the patient experiences progressive cognitive decline but is still able to maintain independent daily activities [5]. This has further led to the usage of the general terms, for example, VCI or VCID, in order to include all cognitive impairments caused by cerebrovascular abnormalities, which could be quite confusing. Essentially, VCI or VCID is used to include a diverse heterogeneous group of cognitive syndromes with vascular origins regardless of the cause of pathogenesis, ranging from mild cognitive impairment to full-blown dementia, and VaD is the most severe stage [3, 8, 18]. The heterogeneity and complexity of VCI however make it difficult for appropriate clinical characterization. Currently there are no accepted pathological criteria for clinical diagnosis for VCI, but identifiable subtypes of VCI have been categorized. This classification system may be useful for clinicians to diagnose for the prevention and the treatment of cognitive dysfunctions. The characterization and classification of VCI are beyond the scope of the present article, and more detailed information could be found in previous reviews [3, 19, 20]. In addition, AD and VaD have traditionally been characterized as separate disorders based on the clinical diagnostic criteria. However, the "pure" form of AD or VaD is not commonly found; instead most people with dementia have mixed pathologies. Besides, AD frequently coexists with cerebrovascular abnormalities such as alterations in vascular structures (e.g., cerebral amyloid angiopathy, CAA) and cerebral infarctions. Accumulating evidence suggested that AD and VaD have additive effects and probably interact with each other. It has been suggested that cerebrovascular dysfunction could play a role in the development and progression of AD. Therefore, the vascular component in the mixed pathology is particularly important for the understanding of the pathogenies of dementia. In the next section, we will give a very brief overview of the major subtypes of VCI. Note that the categorization of VCI described here does not mean to replace any existing clinical criteria for the characterization of different types of VaD but to offer a simplified overview for a better understanding of various VCI terminologies, especially for nonclinician researchers. The terms VCI and VCID are used interchangeable in this article.

2.1. Major Subtypes of Vascular Contributions to Cognitive Impairment (VCI). Many subtypes of VCI or VCID have been described before. The disorder is sometimes classified according to the location of vascular lesions, the causative vascular mechanisms, and their clinical manifestations. Chronic cerebral ischemia and arteriosclerosis were originally thought to be the cause of vascular contributed dementia but later discovered that it is cerebral infarcts rather than ischemia that causes dementia [21]. Therefore, we try to

summarize the major subtypes of VaD according to the type of infarcts exhibited. Similar clinical features of VaD subtypes have been described under different names; therefore we also try to include them into specific groups. In brief, the major subtypes of VCI include vascular mild cognitive impairment (VaMCI), multi-infarct dementia (MID), strategic-infarct dementia (SID) (which is also commonly known as subcortical vascular dementia (SVD) or small vessel disease (SVD)), poststroke dementia, and mixed dementia of AD and VaD. SID (or SVD) accounts for over 40% of all VCI reported [20, 21]. Recent studies also indicated that mixed dementia particularly VaD in conjunction with AD is also commonly observed among elderly people [20, 21].

2.1.1. Vascular Mild Cognitive Impairment (VaMCI). Dementia is preceded by a stage in which mild cognitive decline is first manifested in individuals without affecting care-free daily independent activities. VaMCI is used to describe this intermediate stage between normal cognition and dementia caused by vascular diseases with the presence of cognitive impairment but it is not severe enough to fit into the criteria for VaD [22]. An equivalent stage occurring in AD is referred to as mild cognitive impairment (MCI) and is used to identify people who are at risk for the later amnestic stage of AD. The prevalence of VaMCI is twice as much of VaD [22]. VaMCI is potentially treatable by management of vascular risk factors and diseases [3, 22]. Moreover, VaMCI is usually associated with other diseases such as heart failure, autoimmune disorders, and depression, and previous studies showed that cognitive functions of patients could be improved with respective treatments for these disorders with or without specific treatment for VaMCI [22–24]. VaMCI is common in stroke patients shortly after attacks, and in some patients cognition may improve as part of the stroke recovery process [25, 26].

2.1.2. Large-Vessels VaD: Multi-Infarct Dementia (MID). MID is caused by the "synergistic effects" caused by multiple vascular lesions ("multiple mini strokes") in the brain irrespective of specific location or volume. The mini strokes which disrupted the blood flow to the brain may occur without noticeable clinical symptoms, and over time these lead to irreversible injuries in the brain tissues. MID can be diagnosed by brain imaging techniques such as computed tomography (CT) and magnetic resonance imaging (MRI) scan. MID usually affects people between the ages of 60–75 and is more common in men than in women. However, MID is not the single most common type of VaD in elderly people; instead patients are more likely to have mixed dementia with both AD and VaD pathology (discussed later) [27].

2.1.3. Small-Vessels VaD: Subcortical Ischemic Vascular Dementia (SIVD). SIVD is caused by the occlusion of small penetrating arteries which supply blood to the inner structures of the brain which is recognized as Binswanger's disease, lacunar infarct (LACI), or small vessel disease (SVD). SIVD is also known as silent brain infarction (SBI), and it is the most common type of VaD with 20–40% incidence in our community [28, 29]. This disease can be diagnosed using brain imaging methods, for example, CT and MRI scan. Hypertension is the major risk factor for SVID, and it is therefore potentially preventable and treatable [29]. The hereditary genetic vasculopathy Cerebral Autosomal-Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL), which causes 11% of lacunar stroke cases with leukoaraiosis in middle-aged adults, is one of the best examples of small vessel disease affecting mainly the small penetrating cerebral and leptomeningeal arteries [30].

2.1.4. Strategic-Infarct Dementia (SID). It has been traditionally recognized that single or few focal infarcts occurred in some functionally important brain regions ("strategic") such as lesions in the thalamus, caudate nucleus, lenticular nucleus, angular gyrus, and internal capsule. However, the concept of strategic infarction is under reexamination with larger prospective MRI studies to study the relationship between the extent and location of lesions and the cognitive networks [3].

2.1.5. Poststroke Dementia (PSD). Poststroke dementia (PSD), or poststroke cognitive impairment includes any type of dementia that occurs after stroke, irrespective of the leading causes, which can be vascular, neurodegenerative, or mixed. Having a stroke doubles the risk of dementia development. The prevalence of PSD in patients is about 30%, which varies between age, races, diagnostic criteria, and periods after stroke [31]. The underlying causes of cognitive impairment after stroke are not known at present. It is suggested that vascular lesions caused by ischemia/hypoxia or hemorrhages, cerebral microbleeds, white matter lesions, and neurodegenerative pathologies from other conditions such as AD all contribute to and probably interact with each other to the pathogenesis of PSD. Increasing age is a major determinant of PSD development. It has been reported that 15% of stroke survivors at the age of 60–69 had new-onset dementia, and the prevalence was greatly increased to 26% at the ages 70 to 79 and 36% at the age over 80 years [31–34].

2.1.6. Mixed Dementia. Mixed dementia is a condition in which abnormal features of more than one type of dementia occur simultaneously in the brain, and over 44% of dementia was in fact mixed dementia with a combination of AD and VaD pathologies [35]. The coexistence of AD and vascular lesions is particularly common in older patients. Clinically, a spectrum of vascular diseases is related to the failure of microvasculature functions to regulate cerebral circulation and elimination of interstitial fluid and solutes in both AD and VaD. In AD, abnormal accumulation of amyloid-beta ($A\beta$) in the brain arterial walls, a condition known as cerebral amyloid angiopathy (CAA), leads to the weakening of the brain blood vessel wall and increases the risk of hemorrhages [36]. More recently, dementia research has focused on the importance of the neurovascular unit (NVU) at the level of cellular and molecular mechanisms (discussed later) [6, 30, 37]. Mixed dementia may also include cases of AD and

VaD associated with any other disorders such as Parkinson's disease (PD) and dementia with Lewy bodies (DLB) [35].

The following shows general classification for cognitive impairment with vascular components.

Pathological features includes white matter hyperintensities (WMHs), multiple or single infarcts, hemorrhages, alteration of brain vessel structure, and cerebral hypoperfusion.

Vascular Mild Cognitive Impairment (VaMCI)

(i) Predementia stage: patients have cognitive decline without affecting daily independent functioning

(ii) Symptoms: amnestic, lack of attention, expressive language disorder, and visual depth perception

(iii) Diagnosis methods: brain imaging, cerebrospinal fluid tests, and so forth

The following shows severe cognitive decline.

Vascular Dementia (VaD)

Large-vessel VaD

Multi-infarct dementia (MID)

(i) Caused by multiple infarcts (cortical and/or subcortical) with synergistic effect irrespective of location or volume

(ii) Symptoms: getting lost, language problem, apathy, performing difficulties, emotional lability, and loss of social skills

(iii) Diagnosis methods: a neurological exam, a history of stepwise mental decline, computed tomography (CT) or magnetic resonance imaging (MRI) scans, an electroencephalogram, a transcranial Doppler, and so forth

Small vessel VaD

Subcortical ischemic vascular dementia (SIVD) (may also be known as *Binswanger's disease, Lacunar infarct (LACI), silent brain infarction (SBI), small vessel disease (SVD) (e.g., CADASIL))*

(i) Caused by occlusion of penetrating arteries and thus reduction of blood supply to the brain's deep structures

(ii) Symptoms: sudden hemiparesis, pseudobulbar palsy, small-stepped gait, urinary incontinence, dysarthria, dementia, and changes in effect including inappropriate laughing or crying

(iii) Diagnostic methods: assessment of cognitive deficits (criteria of clinical trial of SIVD), CT or MRI, and so forth

Strategic-infarct dementia (SID)

(i) Caused by focal infarcts in functionally important brain regions (concept of SID is under reexamination)

(ii) Symptoms: mental deterioration, depression, emotional lability, apathy, cognitive, and other deficits

(iii) Diagnostic methods: MRI, CT, perfusion SPECT, and so forth

Poststroke Dementia (PSD)

(i) Including any type of dementia that occurs after stroke irrespective of causes

(ii) Symptoms: trouble with speaking and understanding, trouble with seeing in one or both eyes, cognitive impairment

(iii) Diagnostic methods: CT, PET, functional MRI, spectroscopy, and so forth

Mixed Dementia

(i) Coexistence of more than one type of dementia (e.g., VaD, Alzheimer's disease (AD), and Parkinson's disease (PD))

(ii) Symptoms: symptoms may vary, depending on the type of disease, it may be similar to those of AD or another specific type of dementia

(iii) Diagnostic methods: CT, MRI, and so forth

3. Cerebral Circulation, the Neurovascular Unit (NVU), and VCI

The pathogenesis underlying VCI has remained elusive with many complexities. However, vascular risk factors associated with the cerebral circulation such as hypertension and stroke are shared by many neurodegenerative diseases and are associated with the development of dementia [5]. The cerebral circulation is responsible for one of the most important jobs in the body to provide and regulate blood supply for the highly energy demanding central nervous system. Given the highly perfused nature of the brain, it is not surprising that any disruption or dysfunction in the cerebral circulation will lead to cognitive impairments. The brain is perfused by a sophisticated network of cerebral blood vessels. The anatomy and cellular organization of the cerebral vasculature have been described in detail previously [38, 39] and it is briefly introduced here.

The common carotid arteries carry blood from the heart to the brain and branch into two pairs of carotid arteries (the internal and external carotid arteries). The carotid arteries then give rise to cerebral arteries which further divide into pial arteries that run along the surface of the cortex within the pia-arachnoid. As the pial arteries penetrate into the parenchyma, they become progressively smaller, becoming intracerebral arterioles and cerebral capillaries to supply blood to the corresponding regions of the cerebral cortex. Importantly, the structures and functions of the vessels change significantly as they penetrate into the brain's inner structure. All cerebral vessels have a layer of highly specialized endothelial cells (the blood-brain barrier, BBB) that provide specific barrier functions to regulate the fluid

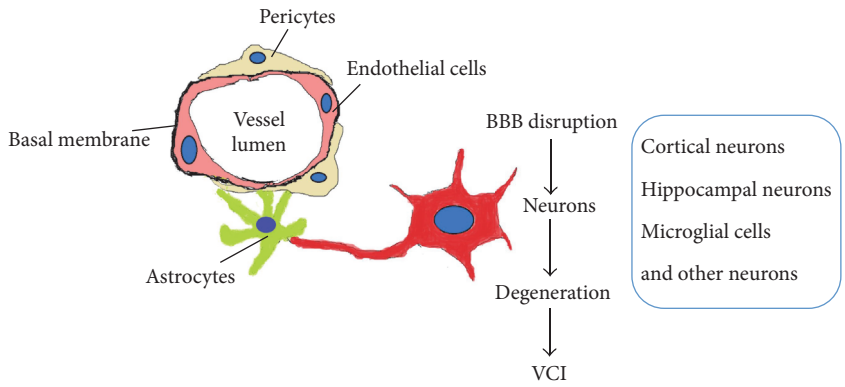

Figure 1: Schematic illustration of the cellular structure of the neurovascular unit (NVU). Dysfunction of the NVU causes blood-brain barrier (BBB) disruption and leads to the degeneration of neurons and VCI.

and solute exchange between the brain and the blood. The pial arteries on the surface of cerebral cortex consist of a layer of endothelial cells surrounded by 2-3 layers of smooth muscle cells and an outer layer of perivascular nerves. The smooth muscle layers are important for supporting the endothelium and regulating vessels contractility, similar to the systemic blood vessels. As the vessels penetrate in the brain and become smaller intracerebral arterioles, the smooth muscle layer becomes thinner and the vessels are completely wrapped around by astrocytic end-feet and other glia cells. The glia (including astrocytes, oligodendrocytes, and microglia) become more and more important in the maintenance and stabilities of the endothelium. As the vessels further progress and become cerebral capillaries, their endothelial cells are sealed by tight junctions, enveloped by pericytes (which replace the smooth muscle layer), and then surrounded by a continuous basement membrane (BM). End-feet of perivascular astrocytes cover the brain capillaries BM and act to integrate the communication between the endothelium and neurons.

The integrated system within a cerebral capillary that is responsible for microvascular homeostasis and neurovascular coupling (the correlation between local neuronal activity and changes in blood flow or hyperemia) is now considered a specialized functional unit known as the neurovascular unit (NVU) (Figure 1). The current understandings of the multicellular organization and functions of the NVU in normal and disease conditions are well described in recent reviews [6, 30, 39]. Essentially, the components of the NVU include all the cells present within the brain capillary (brain endothelial cells, astrocytes, pericytes, microglia, neurons, and circulating inflammatory cells) as well as the basal membrane and endothelial glycocalyx (a network of membrane-bound proteoglycans and glycoproteins covering the luminal-side of the endothelium). It is proposed that vascular risk factors such as hypertension and hypercholesterolemia damage the NVU and lead to chronic hypoperfusion, hypoxia, inflammatory activation, and oxidative stress. The formation of a hypoxic microenvironment at the NVU also directly contributes to inflammatory activation by regulating gene such as hypoxia-inducible factor 1α (HIF1α), which serves as inducer of other genes involved in inflammatory response signaling. Tissue hypoxia and inflammatory activation in neurodegenerative diseases induce the NVU to undergo various cellular interactions and adaptations in responses, exhibiting pathophysiological features such as blood flow perturbation, vascular alterations, adaptive angiogenesis, vascular remodeling, BBB permeabilization, loss of tight junction and/or adherens junction proteins, endothelial injuries, pericytes retraction, BM breakdown, neurovascular uncoupling, and neuronal impairments. This highlights the association of the injury at NVU and the disruption of cerebral microcirculation as a crucial step in ischemic-hypoxic tissue damage leading to neuronal degeneration and cognitive impairment. It is suggested that dysfunction of the NVU may participate in all brain pathologies development, and the primary microvascular events may be etiological to neurological diseases, as well as being involved in diseases manifestation. Epidemiological studies have also provided strong evidence supporting that the NVU dysfunction is closely related to some neurological pathogenesis. For example, hypertension in animal models causing brain vessel rarefaction and a reduction in capillary density and microvessel formation [40]. In the hereditary CADASIL disease of small cerebral arteries, the NVU has been indicated as a predominant causative factor in the pathogenic mechanism of the disease. Structural alterations in the small penetrating cerebral and leptomeningeal arteries in CADASIL caused by pathogenic mutations in NOTCH3 (Notch homolog 3) lead to NVU impairment, causing cerebral blood flow reduction and subcortical ischemia, with lacunar infarcts correlating with cognitive decline [30]. Recently, the NVU has also been proposed to play an important part in the development of cognitive decline observed in AD [30, 37, 41]. It is reported that dementia is more likely to happen when vascular lesions coexist in AD patients. Apart from cerebral amyloid angiopathy (CAA) (the accumulation of amyloid-beta (Aβ) in brain blood vessels), an increased concentration of soluble Aβ peptides in the cerebral circulation of AD patients is also observed [42]. The elevated level of soluble Aβ causes oxidative stress, and the damage on cerebral circulation induced by Aβ precedes cognitive decline, thus suggesting that cerebrovascular dysfunction

might be involved in the pathogenesis of cognitive impairment [42]. All these observations emphasize the importance of the interaction between vascular and neuronal systems in the context of disease pathogenesis and imply the potential of the NVU as a target for disease prevention, treatment, and management.

4. Therapeutic Implications for VCI with Traditional Chinese Medicine (TCM)

Currently there is no standardized treatment regimen for VCI. Several pharmacological agents approved for AD (donepezil, galantamine, rivastigmine, and memantine, Table 1) have been tested to treat patients with VaD with only modest improvements on standard cognitive measures and failed to achieve regulatory approvals [3]. Other antidementia candidates for treating cerebral small vessel disease are also developed (e.g., nimodipine, huperzine) [3]. Uncertainties in diagnostic criteria and the measurements of cognitive domains further pose challenges and hurdles to the progress of drug research and development. Given the fact about the long history of TCM development and uses, many Chinese herbs have been identified for the potential treatment of the dementia-like disorders [43]. VCI belongs to the category of memory disorders according to TCM theories that is known as jian wang (健忘) ("forgetfulness") and dai bing (呆病) ("dementia") [16, 17]. Over the last decades, many clinical trials were conducted in China to investigate TCM for treatment of cognitive impairment and dementia. Despite the enormous efforts involved, most of these trials conducted were not well-designed with limitations such as small sample size, the lack of being truly randomized, double-blind, and placebo-controlled, and the incomplete cognitive assessments dedicated for VaD [44]. Thus, the genuine effectiveness of most TCM therapies assessed for treating dementia remains to be verified. Nevertheless, these studies provided a valuable resource for the search of potential antidementia drugs and therapies [16, 17, 45]. In addition, the most effective approach for VCI treatment is the management of underlying vascular risk factors and prevention of further cerebrovascular injury. Many TCM herbal ingredients have proven abilities to prevent and treat cardiovascular diseases and management of hypertension and stroke. In the following session, the experimental studies of the herbs frequently reported for the treatment of memory disorders with vascular origins are reviewed and discussed (Table 1).

Table 1: Current therapeutic agents and TCM for the treatment against VCI/dementia and their action targets/mechanisms.

Therapeutic agents	Targets/mechanisms for VCI
Donepezil Galantamine Rivastigmine	Acetylcholinesterase (AChE) inhibition
Memantine	Noncompetitive NMDA receptor antagonist
Salvia miltiorrhiza	Antiplatelet aggregation, anti-inflammation, and antioxidative effects
Huperzia serrata	Selective AChE inhibition
Ligusticum chuanxiong	Antiapoptosis, antioxidant, anti-inflammation, antiplatelet, and block calcium (Ca^{2+}) overload
Ginkgo biloba	Restoring mitochondrial dysfunction, improving neuronal energy supplement, improving compromised hippocampal neurogenesis and neuroplasticity, inhibiting Aβ protein aggregation, decreasing blood viscosity, and enhancing microperfusion
Panax ginseng	Antioxidant, antiplatelet, antihyperlipidemic, stimulation of NO production, improvement in blood circulation, and enhancement of vasomotor tone
Saffron	Antioxidant and inhibiting serotonin reuptake in synapses

4.1. Salvia miltiorrhiza. The dried root of *Salvia miltiorrhiza*, also known as Danshen, is a widely used TCM for the treatment and prevention of cardiovascular diseases (CVDs) such as angina pectoris, myocardial infarction, and arteriosclerosis. According to TCM theory, Danshen promotes blood flow and resolves blood stasis. Several registered pharmaceutical products containing Danshen extracts such as Danshen Dripping Pill and Fufang Danshen Tablet are widely used in clinics in the China showing effectiveness for treating CVDs [43]. The herbal extract of Danshen contains water-soluble danshensu (DSS) and salvianolic acid B (Sal B) and lipid-soluble tanshinone I (Tan I), tanshinone IIA (Tan IIA), cryptotanshinone, and dihydrotanshinone. DSS is the major component in Danshen which possesses multiple pharmacological effects that are beneficial to the cardiovascular system, including coronary artery dilatation, antiarrhythmia, microcirculation improvement, protection of myocardial ischemia/reperfusion injury, and antiplatelet aggregation [46–48]. Danshen has been used for the prevention and treatment of cerebral infarction and the underlying mechanisms involved multiple pathways, including antihypotension, antiplatelet aggregation, and anti-inflammatory and antioxidative effects [49]. Recent animal in vivo studies have also shown that DSS exhibited protection against cerebral ischemic/reperfusion injury [50] and neuronal cytotoxicity against oxidative damage [51]. Diabetes is a vascular risk factor shared by AD and VaD, and the spatial learning and memory are impaired in diabetic animals. In cognitive impaired model of streptozotocin-induced diabetic mice, DSS administration ameliorated the learning and memory deficits by attenuating advanced glycation end products-(AGE-) mediated neuroinflammation [52]. The lipid-soluble Tan IIA improved learning and memory deficits in a chronic cerebral ischemia rat model in vivo by protection against free radical insults and regulating the levels of glutamate and γ-aminobutyric acid (GABA) [53]. On the other hand,

Sal B exerted various neuroprotective and anti-inflammatory activities in vivo and in vitro [54].

4.2. Huperzia serrata. An alkaloid isolated from the Chinese herb *Huperzia serrata*, Huperzine A, is a natural cholinesterase inhibitor that selectively inhibits acetylcholinesterase activity and increases acetylcholine levels in brain, thereby improving cognitive functions in patients with dementia [55]. In fact, Huperzine A has been used for treating AD and mild memory deficits since 1994 in the China [55, 56]. In a randomized, double-blinded placebo-controlled clinical trial with the participation of 78 patients who have mild to moderate VaD, Huperzine A treatment significantly improved the cognitive functions in these patients [57]. Furthermore, both animal and human safety evaluations have demonstrated that Huperzine A is safe [58]. Comparing with other acetyl cholinesterase inhibitors such as galantamine, donepezil, and tacrine, Huperzine A showed a longer duration of action, better penetration of the blood-brain barrier, higher oral bioavailability, and fewer adverse reactions [59].

4.3. Ligusticum chuanxiong. Ligusticum chuanxiong is a famous medicinal herb known for its therapeutic effects for treating ischemic stroke and tetramethylpyrazine (TMP) is the major active ingredient. TMP possesses many protective cardiovascular effects, including protection of mitochondrial functions, inhibition of lipid peroxidation by free radicals, attenuating calcium (Ca^{2+}) overload, maintaining calcium homeostasis, inhibiting inflammatory reaction, improving endothelial cell function, and inhibiting myocardial cell apoptosis [60]. Besides, TMP inhibits angiogenesis [61], platelet aggregation, and antithrombosis and ameliorates microcirculation [47, 62]. An injectable formulation known as Guanxinning Injection (or Danshen Chuanxiong Injection) which contains extracts of Danshen and Chuanxiong in combination has displayed a high efficacy in treating hypertension and other cardiovascular diseases [63]. Apart from the beneficial cardiovascular effects, TMP is a potent neuroprotective agent. Evidence from in vivo experiments in rats with middle cerebral artery occlusion (MCAO) demonstrated that TMP has potent neuroprotective effects against ischemic brain injuries caused by transient focal cerebral ischemia reperfusion [64–67]. It has been shown that TMP effectively reduced the size of cerebral infarction and brain edema, suppressed oxidative stress and inflammatory and apoptotic responses [64, 66, 67], and could salvage neurological functions, neuronal dendritic plasticity, and behavioral disturbance in the MCAO stroke model in rats [65, 67]. In another study with scopolamine-induced memory impairment model in rat, TMP could also effectively reverse memory deficits and preserved postsynaptic protein synthesis by restoring the impaired cAMP/PKA/CREB pathways [68]. Besides, TMP attenuated iron-induced oxidative damage and apoptosis in rat cerebellar granule cells [69]. Derivatives of TMP, known as TBN and TN-2, which was synthesized with the addition of powerful free radical-scavenging nitrone moiety (Figure 2), exhibited promising neuroprotective effects. TBN exhibited stronger antioxidative properties without affecting the thrombolytic activity of the parent TMP and produced neuroprotection against ischemic brain injuries in rat transient and permanent MCAO stroke models via the prevention of Ca^{2+}-mediated cellular damage caused by Ca^{2+} influx and overload [70, 71]. Furthermore, TBN protected and rescued dopaminergic neurons from 1-methyl-4-phenylpyridinium- (MPP^+-) and methyl-4-phenyl-1,2,3,6-tetrahydropyridine- (MPTP-) or 6-hydroxyldopamine- (6-OHDA-) induced damage in vitro and in vivo models of Parkinson's disease (PD) by the reduction of oxidative stress plus increased cellular antioxidative defense capacities [72]. TN-2, which has two nitrone moieties, also exhibited potent neuroprotective effects against 6-OHDA-induced neurotoxicity and MPTP/MPP^+-induced dopaminergic neurons damage [73, 74]. Another derivative of TMP, known as T-006 (Figure 2), protected primary cortical neurons from neurotoxicity as well as improvement in memory deficits in APP/PS1 transgenic mice [75].

4.4. Ginkgo biloba. Ginkgo biloba is a widely cultivated tree since ancient China with the sacred belief for its health-promoting properties. The fruits and leaves of Ginkgo biloba are used to treat various types of diseases and symptoms such as atherosclerosis, diabetes, poor circulation, fatigue, vertigo, tinnitus, and cognitive disorder [15]. A standardized extract of Ginkgo biloba (EGb 761) has been shown to exhibit potent antioxidative and antiplatelet effects and could effectively reduce damage from cerebral ischemia [76–81]. Besides, EGb 761 extract protected hippocampal neuronal and neuroglia cells against damage and enhanced the recovery of learning/memory impairments from cerebral ischemia/reperfusion in mice and rats [79–83]. Furthermore, the Ginkgo extract has been reported to improve memory deficits and cognitive dysfunctions in various experimental models of memory disorders such as chronic stress, aging, and Parkinson's disease (PD) [84–86]. Recently, EGb 761 has also been shown to enhance the functions and integrity of cerebral microvascular endothelial cells under chronic stress induced by hypoxia, hyperglycemia, or amyloid-beta ($A\beta$) protein [87, 88], suggesting that functions of the neurovascular unit might play an important role in the protective effects of EGb 761. Interestingly, various clinical trials have reported the efficacy of EGb 761 in improving the cognitive impairment of patients with AD and VaD [89–92]. In a recent study of a 24-week randomized controlled trial [92], once-daily preparation of EGb 761 was shown to improve cognitive functions, neuropsychiatric symptoms, and functional abilities in 333 patients with AD and 71 with VaD. In the market, Ginkgo extract is developed into an herbal supplement called Gingium® which is for improving mild to moderate age-related cognitive impairments and alleviating symptoms of AD, VaD, and/or mixed dementia. It is noted that despite the encouraging evidence of the beneficial effects of Ginkgo biloba consumption in treating VaD, the potential drug-drug interactions especially associated with long-term usage of Ginkgo extract required further evaluation.

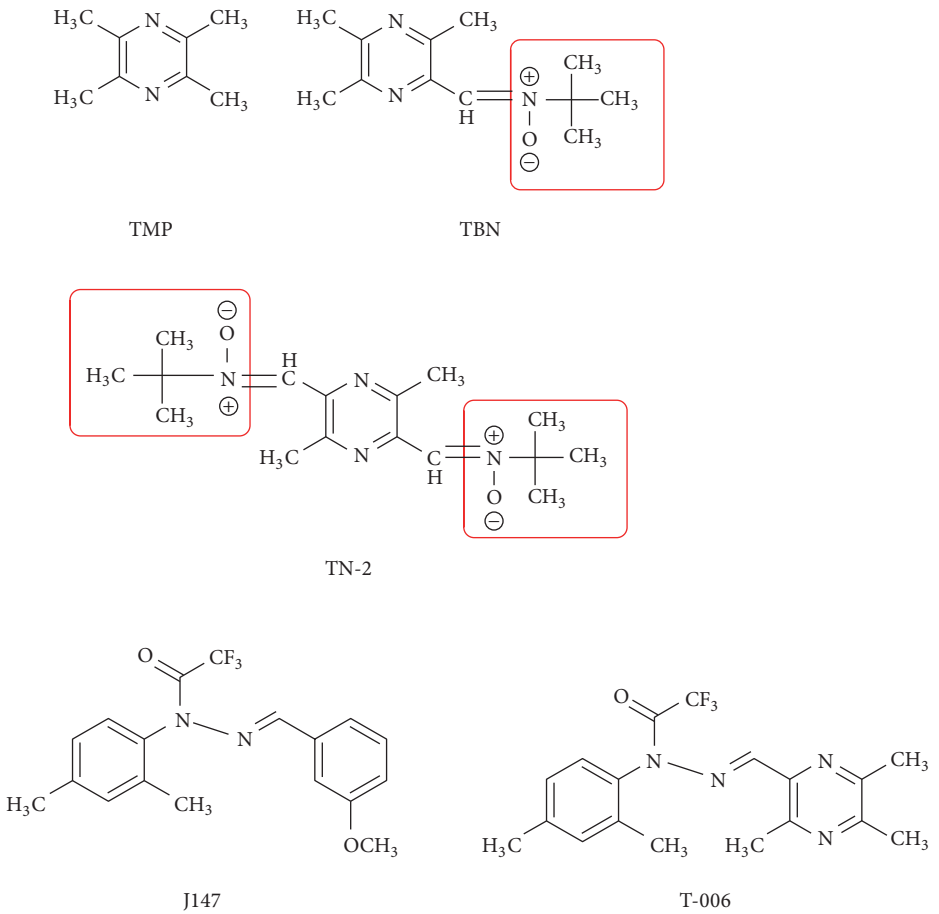

Figure 2: The structures of derivatives of TMP.

4.5. Panax ginseng. The root of the Panax ginseng is well-known for many health benefits and has been widely used to promote physical strength and healthy minds. Ginseng and its active pharmacological ingredients ginsenosides have been shown to improve age-related memory and cognitive deficits; their therapeutic effects for the treatments of cerebrovascular diseases and neurodegenerative diseases have been reviewed recently [93–95]. Several studies reported that total ginseng, total saponins, and Rg1, Rb1, and Rg2 have neuroprotective effects [96–99]. Rg1 promoted the proliferation of hippocampal progenitor cells after transient global ischemia experimental brain ischemia [99], whereas Rb1 has been reported to protect hippocampal neurons against ischemia [100]. In a recent study using a VaD rat model, Rg2 improved neurological performance and memory ability of VaD rats after cerebral ischemia reperfusion through the modulation of antiapoptotic signaling pathways [98]. Results from the experimental AD models have also demonstrated that ginseng extract and ginsenosides are effective in protecting and alleviating neuronal damage [93]. Two clinical studies on ginseng therapy for the treatment of AD reported a significant improvement in cognitive performance [101, 102].

4.6. TCM Formulae for Treatment of VaD. In recent years, various TCM formulae for the treatment of VaD have been developed, for example, Sailuotong capsule (SLT) and Fufangdanshen tablets (FFDS) [103, 104]. SLT is composed of ginseng extract (ginseng total saponins), Ginkgo biloba extract (Yinxingtong ester), and saffron extract (saffron total glycosides). The therapeutic functions of SLT are yi-qi-huo-xue and hua-yu-tong-luo according to the TCM theory. Pharmacodynamics studies showed that SLT could significantly improve neurological symptoms caused by focal cerebral ischemia and improve learning and memory ability in animal models of VaD. In healthy adults, one-week consumption of SLT improved both neurocognitive and cardiovascular functions [103]. More recently, an international multicenter phase II clinical trial of SLT in patients with mild to moderate VaD has been initiated in 2012 to 2014, and the clinical results/outcomes will be available soon [105]. In recent phase III clinical trial launched in 2016, which involved research teams of Xiyuan Hospital of the China Academy of Chinese Medical Sciences and the National Institute of Complementary Medicine at Western Sydney University, over 200 Australians with dementia were recruited and participated in this clinical trials, and results obtained from the pilot studies have implicated the effectiveness of SLT uses in improving learning and memory [106]. On the other hand, an ongoing double-blind, randomized, parallel placebo-controlled clinical study on the evaluation of efficacy and safety of FFDS (*Radix*

Salvia miltiorrhiza formula tablets) for patients with mild to moderate VaD has shown encouraging data on treating cognitive symptoms in VaD patients [104].

5. Conclusions

VCI (or VCID) is a complex form of dementia, involving both vascular and neurological aspects. The heterogeneity of VCI and the coexistence of other forms of dementia such as AD make it even harder for effective drug development and clinical treatment. Recently, the important roles of the neurovascular unit (NVU) in neurodegenerative diseases are rigorously studied. Unfortunately, there is no standard therapy for VCI. Most Western medicines are target-oriented whereas TCM offers therapeutic outcomes via holistic approaches which are probably appropriate for treating VCI which involved various factors. Evidence-based TCM therapies have shown some promising results for treating the symptoms of cognitive impairments. Therefore, TCM serves as a rich resource for therapeutic developments for treating dementia, and it will be of interest to further investigate the actions of potential TCM ingredients on the functions of NVU.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

The Effects of Four-Week Multivitamin Supplementation on Mood in Healthy Older Women: A Randomized Controlled Trial

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Objective. Nutritional deficiencies have been associated with cognitive decline and mood disturbances. Vitamin intake can influence mood and randomized controlled trials have demonstrated that multivitamin supplements are capable of reducing mild symptoms of mood dysfunction. However, few studies have focussed on healthy older women. *Methods*. This study investigated the effects of four weeks' multivitamin supplementation on mood in 76 healthy women aged 50–75 years. Mood was assessed before and after intervention in the laboratory using measures of current mood and retrospective experiences of mood over the past week or longer. Mobile phones were used to assess changes in real-time mood ratings, twice weekly in the home. *Results*. There were no multivitamin-related benefits identified for measures of current mood or reflections of recent mood when measured in the laboratory. In-home assessments, where mood was rated several hours after dose, revealed multivitamin supplementation improved ratings of stress, with a trend to reduce mental fatigue. *Conclusions*. Over four weeks, subtle changes to stress produced by multivitamin supplementation in healthy older women may not be detected when only pre- and posttreatment mood is captured. In-home mobile phone-based assessments may be more sensitive to the effects of nutritional interventions compared to traditional in-laboratory assessments.

1. Introduction

Vitamin insufficiency is common amongst older people [1, 2] and can lead to detriments to neurological function [3]. For instance, suboptimal intake of vitamins and minerals including folate, vitamin B6, vitamin B12, and zinc has been associated with mild psychiatric symptoms [4] and mood disorders in older people [5, 6]. In addition to psychiatric disturbances, deficiency of selected nutrients including folate and vitamin B12 has also been implicated in dementia [7]. Depression and anxiety represent significant risk factors for cognitive decline and dementia [8, 9]. Importantly, these dementia risk factors are modifiable and can serve as potential targets for intervention.

Multivitamins contain a range of B vitamins, as well as antioxidant vitamins and minerals which exert effects on the central nervous system including synthesis of catecholamine neurotransmitters and serotonin [10, 11]. The potential for multivitamins to modify mood has been demonstrated in a meta-analysis of eight randomized controlled trials (RCTs) which indicated that multivitamin use over a period of ≥4 weeks can reduce mild symptoms of mood dysfunction in healthy people, free from clinical mood disorders [12]. Only one of these trials focussed exclusively on people over the age of 50 years [13], demonstrating that, in men aged 50–70 years, eight weeks' supplementation with a multivitamin mineral and herbal (MVMH) formula improved ratings of current mood as well as reflections of mood over the preceding week.

It has been suggested that micronutrient interventions should show larger effects on mood when measured using near-to-real-time assessments, which permit mood to be tracked on a momentary basis, than when assessed by

measures which rate experiences of mood over the past week or month [14]. This may be due to effects of micronutrients on neurotransmitters, as neurotransmitter and hormonal influences have more direct associations with immediate mood responses compared to delayed reflections on mood based on a longer time period [14, 15]. Our recent examination of acute effects of multivitamin supplementation conducted in women aged 50–75 years demonstrated benefits to general mood and perceived stress one to two hours after MVMH supplementation [16]. These findings indicate that, in older people, mood improvements can arise in the hours following multivitamin supplementation. Therefore a supplementation period of several months may not be necessary to detect mood improvements in older people due to multivitamin supplementation. For this reason there is a need to identify mood measures that employ a suitable time scale to best detect mood changes in shorter term intervention trials.

Ecological momentary assessment (EMA) is a methodology which enables an individual to report on symptoms, mood, and other behavioural parameters close in time to experience, and these reports are obtained many times over the course of a study [17]. EMA can provide real-time measures of mood and affords greater assessment of dynamic processes (i.e., change in mood over time) than laboratory based assessments [18]. There is emerging evidence that EMA methods may be particularly sensitive to the mood enhancing effects of multivitamin supplementation. In healthy young people, four weeks' multivitamin supplementation resulted in increased mental stamina, physical stamina, and concentration when mood was rated weekly on mobile phone devices [19] and four months' multivitamin supplementation improved ratings of current stress, physical fatigue, and anxiety using similar methodology [20]. By contrast, there were no benefits of multivitamins to mood or general well-being when assessments were conducted in a laboratory setting using traditional pretreatment/posttreatment mood measurement [20]. While these studies measured mood at multiple time points outside of the laboratory setting, the rates of mood changes were not examined in a continuous manner. To date, no RCTs have utilised EMA methodology to investigate the time course of multivitamin-related mood changes in older people.

This investigation extends our examination of acute effects of multivitamin supplements in older women [16] and reports the results of a longer four-week supplementation period in the same participant sample, using the same MVMH preparation. Our analysis from the acute time point identified immediate mood improvements, particularly improvements to stress, one to two hours after MVMH intake [16]. In the current study mood was assessed in the laboratory using standard mood measures which rated retrospective experiences of mood over the past week or longer, as well as using assessments which rated current experiences of mood. We set out to examine whether ratings of current mood, including energy levels, alertness, stress, and anxiety, would be more sensitive to the effects of four weeks' multivitamin mineral and herbal (MVMH) supplementation compared to retrospective mood measures. A further aim of this study was to assess real-time mood changes due to the MVMH supplements over the four-week period. To achieve this purpose, mood was rated in the home, twice weekly using mobile phones.

2. Methods

2.1. Trial Design and Randomization. This study followed a double-blind, placebo-controlled, parallel group, randomized design. Participants were allocated to receive either the MVMH formula or a placebo matched for appearance, smell, and taste. Participants were randomized in blocks of 4, with a ratio of 1 : 1 using a computer generated sequence. Randomization was implemented by personnel not involved in the trial, using sequentially numbered treatments. The study protocol was approved by the Swinburne University of Technology Human Research Ethics Committee (SUHREC) and was carried out in accordance with the Declaration of Helsinki. All participants signed a consent form prior to enrolment in the trial. The trial is registered as the "Behavioural Effects of Multivitamin Supplements" study on the Australian New Zealand Clinical Trials Registry (ACTRN12613001087741). Data collection took place between November 2013 and July 2014.

2.2. Participants. The sample consisted of 76 community dwelling females aged 50–75 years (M = 63.6 years, SD = 6.4 years), who were not currently engaged in full-time employment. We focussed on this age range as previous work by our group has identified mood benefits due to multivitamin supplementation in men aged 50–70 years [13], but relevant research in older women is lacking. Participants were recruited from the community using an existing database and via newspaper and post advertisements. All participants were in good health, English speaking, nonsmokers, and free from diabetes, cardiovascular disease, dementia, stroke, and other neurological conditions. Further exclusion criteria included a history of head trauma, alcohol abuse, clinically diagnosed depression, anxiety, or other psychiatric disorders, and use of antidepressant medication, antianxiety medication, anticholinergic drugs, acetylcholinesterase inhibitors, or high-dose anticoagulants. Participants who received a score below 25 on the Mini Mental State Examination (MMSE) [21] were ineligible as this score may indicate the presence of cognitive decline. All participants were required to abstain from using vitamin E, multivitamins, vitamin B complex, ginkgo biloba, fish oils, and St John's Wort supplementation for 4 weeks preceding the first study visit and for the duration of the study.

2.3. Interventions. The MVMH supplement was Swisse Women's 50+ Ultivite (Australian Register of Therapeutic Goods ID: 187121). The study treatment was given orally in tablet form. Supplements were packaged in blister packs containing 7 supplements labelled with each day of the week. Each participant received an opaque box containing 5 blister packs (35 supplements). Participants were required to take one tablet daily with breakfast for 4 weeks (~30 supplements). Table 1 lists the ingredients of the MVMH supplement. As shown in Table 1 the majority of the non-MVM

Table 1: Ingredients of Swisse Women's 50+ Ultivite formula.

Component	Daily dose
Retinyl acetate (equiv. to 2500 IU of vitamin A)	862.5 μg
D-Alpha-tocopheryl acid succinate (equiv. to vitamin E 30.25 IU)	20 mg
Thiamine hydrochloride (vitamin B_1)	30 mg
Riboflavin (vitamin B_2)	30 mg
Nicotinamide (vitamin B_3)	20 mg
Calcium pantothenate (vitamin B_5) (equiv. to pantothenic acid 68.7 mg)	70 mg
Pyridoxine hydrochloride (vitamin B_6) (equiv. to pyridoxine 20.56 mg)	30 mg
Cyanocobalamin (vitamin B_{12})	115 μg
Cholecalciferol (vitamin D_3) (equiv. to vitamin D 200 IU)	5 μg
Biotin (vitamin H)	150 μg
Folic acid	500 μg
Calcium ascorbate dihydrate (vitamin C) (equiv. to ascorbic acid 165.3 mg)	200 mg
Phytomenadione (vitamin K)	60 μg
Zinc amino acid chelate (equiv. to zinc 20 mg)	75 mg
Calcium orotate (equiv. to calcium 10 mg)	100 mg
Magnesium aspartate dihydrate (equiv. to magnesium 6.74 mg)	100 mg
Selenomethionine (equiv. to selenium 26 mcg)	65 μg
Molybdenum trioxide (equiv. to molybdenum 45 μg)	67.5 μg
Chromium picolinate (equiv. to chromium 50 μg)	402 μg
Manganese amino acid chelate (equiv. to manganese 4 mg)	40 mg
Ferrous fumarate (equiv. to iron 5 mg)	16.01 mg
Copper gluconate (equiv. to copper 1.7 mg)	8.57 mg
Potassium iodide (equiv. to iodine 149.83 mcg) (equiv. to potassium 46.18 mcg)	196 μg
Lactobacillus rhamnosus	80 million organisms
Lactobacillus acidophilus	80 million organisms
Bifidobacterium longum	35 million organisms
Citrus bioflavonoids extract	20 mg
Vaccinium macrocarpon fruit dry (patented cranberry Pacran)	800 mg
Silybum marianum dry fruit (St. Mary's thistle) (equiv. to flavanolignans calculated as silybin 17.14 mg)	1500 mg
Ginkgo biloba leaf dry (maidenhair tree) (equiv. to ginkgo flavonglycosides 4.8 mg and ginkgolides and bilobalide 1.2 mg)	1000 mg
Turnera diffusa leaf dry (damiana)	500 mg
Scutellaria lateriflora herb dry (skullcap)	50 mg
Vitis vinifera dry seed (grape seed) (equiv. to procyanidins 7.9 mg)	1000 mg
Urtica dioica leaf dry (nettle)	100 mg
Ubidecarenone (Coenzyme Q10) (from patented ultrasome CoQ10)	2 mg
Cynara scolymus leaf dry (globe artichoke)	50 mg
Cimicifuga racemosa root & rhizome dry (black cohosh)	200 mg
Curcuma longa rhizome dry (turmeric)	100 mg
Withania somnifera root dry (ashwagandha)	500 mg
Crataegus monogyna fruit dry (hawthorn)	100 mg
Silica colloidal anhydrous (equiv. to silicon 9.35 mg)	20 mg
Bacopa monnieri whole plant dry (*Bacopa*) (equiv. to bacosides calculated as bacoside A 1.125 mg)	50 mg
Lecithin powder-soy phosphatidylserine enriched soy (equiv. to phosphatidylserine 2 mg)	10 mg
Spearmint oil	2 mg
Vaccinium myrtillus fruit dry (bilberry) (equiv. to anthocyanosides 324 mcg)	100 mg
Tagetes erecta flower dry (marigold) (lutein esters calculated as lutein (of *Tagetes erecta*) 1 mg)	100 mg

ingredients are at subtherapeutic levels with the exceptions of ashwagandha, ginkgo, and grape seed. The placebo tablets contained starch and a small amount of riboflavin (2 mg) to give them a similar smell and colouration of the urine. Participants were required to abstain from the treatment on the day of posttreatment testing. Treatment compliance was determined using a daily tablet taking log and by counting remaining tablets at the posttreatment assessment.

2.4. Sample Size. A meta-analysis [12] has indicated that multivitamin formulas, with comparable doses of B vitamins to the current study, exert small to medium sized effects (standard mean difference = .29) on mood measures including the General Health Questionnaire. Power analysis was conducted using G*Power 3.1.3. To have 80% chance of detecting an effect size of this magnitude ($F = .15$) in a two-armed study (multivitamin, placebo) with at least 3 time points it was determined that a total sample of 75 participants would be required (alpha level = .05).

2.5. Procedure. Potential participants were screened over the telephone to determine initial eligibility. Those who fulfilled the eligibility criteria were invited to attend the first session where additional screening and baseline and acute data (published elsewhere, [16]) were collected.

2.5.1. Baseline Visit. Participants attended the baseline laboratory based assessment between 0900 and 1100 hours. On the day of the baseline visit participants were asked to refrain from caffeine ingestion and to consume their "usual" breakfast. The participant's breakfast was recorded and they were requested to consume the same breakfast on the day of the posttreatment study visit. At the baseline visit, participants provided informed consent and completed a medical health questionnaire and the MMSE prior to enrolment in the study. Participants completed baseline retrospective mood ratings and current mood ratings using a mobile phone device, as well as the cognitive assessments, prior to being randomized to receive the MVMH formula or placebo. Participants completed a brief food frequency questionnaire which assessed general intake of 29 different foods over the past 12 months. Daily serves of fruit and vegetable intake were scored on a 6-point Likert scale from 0 (none) to 5 (4 or more).

2.5.2. Mood Assessments in the Home. Participants were provided with a mobile phone device and were instructed to rate their current mood using Visual Analogue Scales on two days each week for the four-week period. Participants were asked to ensure there were at least two days separating each mood report and that mood reports were completed after MVMH intake for that day. Participants were requested to complete the mood assessments at 1000 or 1500 hours and to ensure they completed the assessments at both times across the intervention period.

2.5.3. Posttreatment Visit. Participants returned for their post treatment appointment 4 weeks later at the same time of day as their baseline visit. All mood measures were repeated, with exception of the 1-hour postdose assessment.

2.6. Measures

2.6.1. Ratings of Current Mood. The State-Trait Anxiety Inventory-State (STAI-S) assesses intensity of individual's current state of anxiety using 20 items [22]. Scores range from 20 to 80 with higher scores indicating greater anxiety. Participants also rated their current mood using scales presented on mobile phone devices both during the study visits and at home. The Bond Lader Visual Analogue Scales (VAS) [23] were used to assess feelings of alertness, contentedness, and calmness. Participants marked the position of their current subjective state on a horizontal line anchored at either end by adjective pairs (e.g., happy-sad). Each line was scored as the percentage of the total distance from the negative anchor, with higher scores indicating more positive mood states. The alertness, contentedness, and calmness subscales were calculated from 16 adjective pairs. Additional VAS measures were used to assess current levels of stress, anxiety, concentration, physical fatigue, and mental fatigue on lines with end-points labelled "Not at all" and "Extremely." Each scale provided a single subjective score between 0 and 100, with lower scores indicative of more desirable mood states on the mood scales and higher energy levels on the fatigue scales. Higher scores on the concentration item were indicative of greater ability to concentrate.

2.6.2. General Health Questionnaire. Participants completed a number of pen-and-paper measures designed for use in nonclinical samples. The General Health Questionnaire-28 (GHQ-28) [24] assesses general mild psychiatric symptoms experienced over the past week using 28 items relevant to health-related quality of life. In nonclinical samples, improved mood ratings have been observed on the GHQ following multivitamin supplementation of ≥28 days [12]. Scores on the GHQ-28 range from 0 to 84, with lower scores indicating better health-related quality of life. Participants completed the General Health Questionnaire (GHQ) at an additional 2-week time point in the home and returned the questionnaire via mail.

2.6.3. Additional Retrospective Mood Measures. All other pen-and-paper measures were completed at baseline and posttreatment study visits only. The Hospital Anxiety and Depression Scale (HADS) [25] is a commonly used measure designed to screen for mood disorders in general (nonpsychiatric) medical outpatients. The HADS provides a brief measure of anxiety and depression experienced over the past week. Scores on each subscale range from 0 to 21, with higher scores indicating more severe anxiety or depression. The Perceived Stress Scale (PSS) [26] was used to measure the degree to which respondents viewed situations which occurred over the past month as stressful. Scores on the PSS range from 0 to 40 with higher scores indicating higher levels of perceived stress. The Chalder Fatigue Scale [27] was used to measure severity of symptoms relating to physical and mental

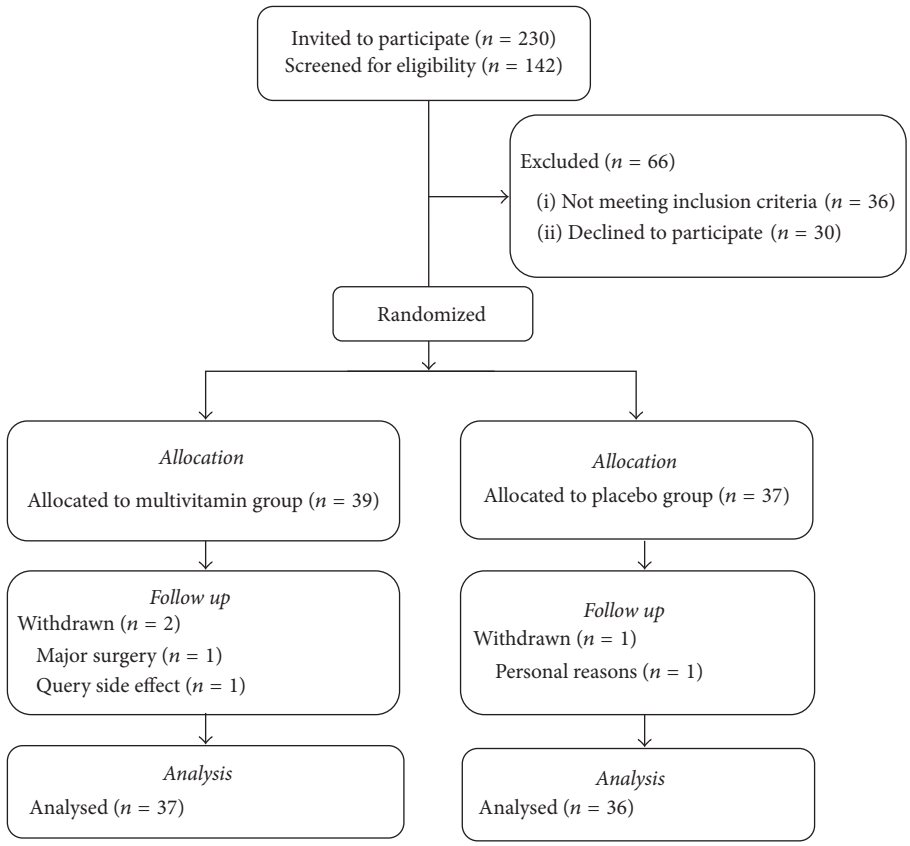

Figure 1: Recruitment and retention flowchart.

fatigue experienced over the past week. The scale consists of 14 items. Scores range from 0 to 42, with higher scores indicating greater fatigue.

2.7. Analysis. Statistical analyses were carried out in SPSS version 22. Independent groups t-tests were used to examine baseline group difference in age, body mass index, and education. Multivariate analysis of variance (MANOVA) was used to examine baseline group differences in mood.

Education was controlled for when examining all mood outcomes; 2 (treatment: multivitamin, placebo) × 2 (time: baseline, posttreatment) repeated mixed methods MANCOVA was conducted for the VAS and STAI measures of current mood.

A 2 (treatment: multivitamin, placebo) × 3 (time: baseline, 2 weeks, and posttreatment) MANCOVA was used for the analysis of the subscales of the GHQ-28. To avoid multicollinearity the total GHQ score was analysed in a separate ANCOVA model. An additional 2 (treatment: multivitamin, placebo) × 2 (time: baseline, posttreatment) MANCOVA was conducted for the Chalder Fatigue Scale, HADS, and PSS.

Secondary analysis was conducted on data from the EMA mobile phone VAS assessments using logistic longitudinal multilevel modelling in HLM7. This analysis was conducted to examine the dynamic effects of the MVMH formula on mood over time. The VAS scores were highly clustered around two distinct values, indicating they were not suitable for transformation. As these values represented the default low and high scores in the mobile phone program, VAS scores were recoded in binary form as below the midpoint ≤50 or above the midpoint >50. The default scores refer to the scores obtained on the scale if the participant pressed the left button first (score of 24 out of 100) or right button first (score of 76 out of 100).

Approximately eight time points were included in the multilevel analysis for each participant, commencing at the baseline laboratory session and concluding at the final in-home assessment.

3. Results

Participants were 39 women allocated to the multivitamin group and 37 allocated to placebo group. The participant recruitment flowchart is shown in Figure 1. Three participants withdrew during the course of the study leaving a total of 37 individuals in the multivitamin group and 36 in the placebo group (4% withdrawal rate).

Demographic details of the participant sample are shown in Table 2. Details of medication use have been reported elsewhere [16]. Independent t-tests indicated there were no significant group differences in participant age or body mass index (kg/m^2); however participants assigned the MVMH treatment had completed significantly more years of education ($t(74) = 2.19$, $p < .05$). The majority of participants were

Table 2: Participant demographics at baseline.

Characteristic	Multivitamin M (SD)	Placebo M (SD)
Age	64.4 (6.3)	62.8 (6.4)
Body mass index	24.4 (3.5)	26.3 (5.0)
Years of education	17 (3.4)	15.4 (3.3)*
Employed part time (% of yes)	36%	41%
Retired (% yes)	44%	49%

*$p < .05$.

either retired (46%) or working part time/casual hours (38%). The majority of participants reported consuming 2 serves of fruit per day (56% multivitamin group, 57% placebo group) and 3 serves of vegetables (49% multivitamin group, 33% placebo group). Chi square tests of independence indicated there were no interactions between treatment group and daily portions of fruit ($\chi^2(4) = 2.91$, $p = .57$) or vegetable intake fruit ($\chi^2(4) = 4.22$, $p = .38$). Average compliance for both groups was 99% (SD = 3), with no participants dropping below 89% adherence. On average participants completed 7 (SD = 1.2) mood assessments in the home using the mobile phone device. MANOVA analysis indicated at baseline there were no significant group differences between mood ratings on the GHQ measures ($F(4, 71) = .66$, *Wilk's* $\lambda = .96$, and $p = .62$), visual analogue measures, and STAI ($F(9, 66) = .85$, *Wilk's* $\lambda = .90$, and $p = .58$) or other retrospective mood measures ($F(5, 70) = 1.73$, *Wilk's* $\lambda = .89$, and $p = .14$).

3.1. Laboratory Mood Assessments. Baseline and posttreatment mood scores are shown in Table 3. In terms of the effect of the MVMH formula on mood, there was no significant time × treatment interaction for the GHQ-28 total score ($F(2, 128) = .78$, $p = .46$). A significant time × treatment interaction was identified for the GHQ-28 subscores ($F(8, 57) = 2.27$, *Wilk's* $\lambda = .76$, and $p = .04$). However, the univariate time × treatment interaction was not significant for any of these measures in isolation. There were no significant time × treatment interactions for the Bond Lader VAS, additional VAS, and STAI ratings of current mood ($F(9, 60) = .97$, *Wilk's* $\lambda = .87$, and $p = .50$). Similarly there were no significant time × treatment interactions for the other retrospective mood scales including the Chalder Fatigue total score and subscales, the HADS, and the PSS ($F(5, 65) = .94$, *Wilk's* $\lambda = .93$, and $p = .47$).

3.2. EMA Assessments of Current Mood in the Home. In total 54% of mood ratings were completed in the morning, with 40% completed in the the afternoon and 6% in the evening. This was highly comparable for both treatment groups. Slope and odds ratio values for each group are shown in Table 4 while controlling for years of education. A significant interaction between time and treatment was identified for stress ($t(71) = 2.82$, $p = .006$) and so was a very nearly significant interaction effect for mental fatigue ($t(71) = 1.957$, $p = .054$). Odds ratios indicate the daily reduction in stress was on average 5.3% greater in the multivitamin group than the placebo and reduction in mental fatigue was on average 3.7% greater in the multivitamin group than in the placebo group. Changes in stress and mental fatigue ratings, concluding at the final in-home assessment, are shown in Figures 2 and 3, respectively.

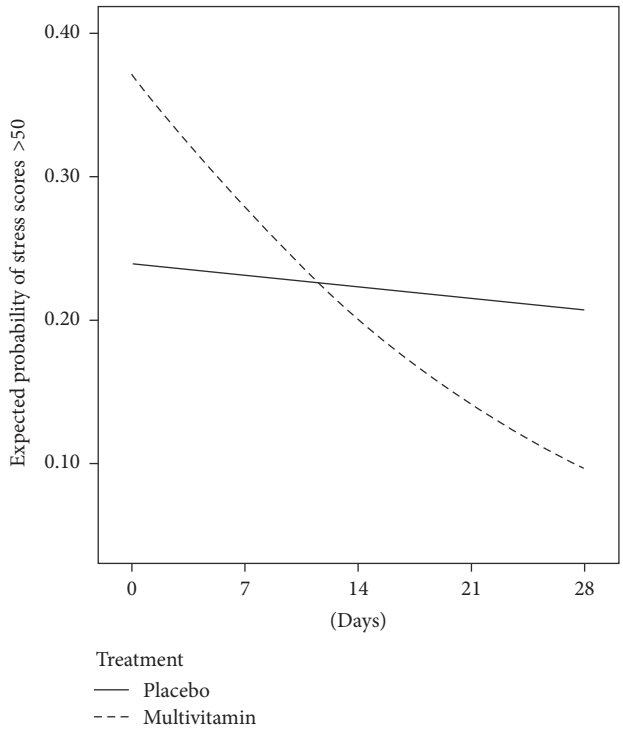

Figure 2: Change in the probability of stress ratings >50, assessed biweekly using mobile phone devices, commencing at baseline and concluding at the final in-home assessment for someone with 12 years of education.

4. Discussion

This study of healthy older women found no benefit of four weeks' MVMH supplementation to ratings of retrospective or current experiences of mood. However, improvements to stress and a trend for mental fatigue to be reduced were observed when mood was rated in the home on multiple occasions, using mobile phone devices. These results differ from trials which have demonstrated that a period of 4 weeks' multivitamin supplementation is sufficient to induce mood benefits to similar retrospective measures in healthy younger adults [28, 29]. However findings of mood benefits detected outside the laboratory are consistent with results of other researchers who have utilised comparable mobile phone based assessments [19, 20].

The present study did not identify multivitamin-related benefits to measures of current mood or retrospective mood, when rated in the laboratory. Improvements to both categories of mood ratings on several of the same measures included in the present study, including the GHQ and VAS mood scales, have previously been found to benefit from multivitamin supplementation in men aged 50–69 years, over

Table 3: Means and standard deviations for the mood assessments at baseline and 4 weeks after treatment.

Measure	Group		Baseline		Midpoint		Posttreatment	
		N	M	SD	M	SD	M	SD
General Health Questionnaire								
Total	Multivitamin	35	15.06	6.65	14.31	5.86	14.47	5.48
	Placebo	33	15.24	6.74	13.00	6.01	12.79	5.84
Somatic	Multivitamin	35	3.60	2.76	4.03	3.22	4.29	3.04
	Placebo	33	4.33	3.01	3.64	3.20	3.21	2.38
Anxiety	Multivitamin	35	3.57	2.76	3.42	2.52	3.20	2.58
	Placebo	33	3.48	2.58	2.97	2.56	3.00	2.52
Social dysfunction	Multivitamin	35	6.83	1.67	6.23	1.68	6.51	1.40
	Placebo	33	6.82	1.89	5.89	1.58	6.12	1.52
Depression	Multivitamin	35	1.06	2.09	0.63	1.33	0.46	1.12
	Placebo	33	0.61	1.52	0.52	1.56	0.45	1.50
Chalder Fatigue Scale								
Total	Multivitamin	37	16.00	4.33			13.35	3.22
	Placebo	36	14.47	3.75			13.03	3.20
Physical	Multivitamin	37	8.83	2.63			7.47	1.66
	Placebo	36	8.11	2.61			7.31	2.19
Mental	Multivitamin	37	7.05	2.17			5.62	1.40
	Placebo	36	6.36	1.53			5.72	1.39
Hospital Anxiety and Depression Scale								
Anxiety	Multivitamin	37	4.46	2.30			3.81	2.08
	Placebo	36	4.33	2.69			4.03	3.15
Depression	Multivitamin	37	2.16	2.10			1.35	1.57
	Placebo	36	2.23	1.75			1.42	2.00
Perceived Stress Scale								
	Multivitamin	37	19.05	3.21			18.70	1.90
	Placebo	35	18.40	2.08			17.74	2.00
State-Trait Anxiety Inventory								
State version	Multivitamin	37	32.81	10.27			28.32	6.85
	Placebo	35	30.54	9.09			27.06	9.05
Visual Analogue Scales								
Alertness	Multivitamin	37	68.52	17.51			72.89	16.52
	Placebo	35	70.83	16.19			75.62	17.96
Contentedness	Multivitamin	37	77.57	16.88			81.55	13.89
	Placebo	35	81.47	16.32			83.45	16.58
Calmness	Multivitamin	37	66.43	21.74			74.09	16.66
	Placebo	35	74.26	18.92			77.20	19.52
Stress	Multivitamin	37	21.76	21.61			12.38	13.63
	Placebo	35	14.77	17.81			14.09	17.15
Concentration	Multivitamin	37	65.95	28.81			72.62	20.40
	Placebo	35	61.83	26.65			66.89	29.76
Anxiety	Multivitamin	37	16.24	19.15			12.97	15.86
	Placebo	35	14.60	19.24			10.69	14.27
Mental fatigue	Multivitamin	37	23.24	24.14			18.22	20.91
	Placebo	35	23.77	21.98			19.17	19.24
Physical fatigue	Multivitamin	37	21.19	23.19			20.05	23.03
	Placebo	35	17.74	18.89			19.09	20.74

N = represents participants included in the pretreatment to posttreatment analysis.

Table 4: Fixed effects coefficients and daily odds ratios for mobile phone ratings of mood over the intervention period controlling for years of education.

Mood rating	Treatment	*Daily odds ratio*		*Odds ratio test*		*Test difference odds ratios*	
		Estimate	95% CI	$t(71)$	p value	$t(71)$	p value
Alertness	Multivitamin	1.052	(1.021, 1.084)	3.391	**.001**	1.300	.198
	Placebo	1.023	(.992, 1.055)	1.484	.142		
Contentedness	Multivitamin	1.008	(.988, 1.029)	.832	.408	.483	.631
	Placebo	1.017	(.990, 1.044)	1.241	.219		
Calmness	Multivitamin	1.044	(1.020, 1.067)	3.781	**<.001**	−.550	.584
	Placebo	1.035	(1.014, 1.056)	3.416	**.001**		
Stress	Multivitamin	.941	(.918, .965)	−4.802	**<.001**	2.820	**.006**
	Placebo	.994	(.965, 1.023)	−.442	.660		
Concentration	Multivitamin	1.022	(.995, 1.051)	1.605	.113	.220	.827
	Placebo	1.026	(1.002, 1.052)	2.133	**.036**		
Anxiety	Multivitamin	.957	(.930, .984)	−3.147	**.002**	.855	.395
	Placebo	.973	(.947, .999)	−1.630	.107		
Mental fatigue	Multivitamin	.928	(.900, .956)	−4.941	**<.001**	1.957	**.054**
	Placebo	.965	(.940, .99)	−2.802	**.007**		
Physical fatigue	Multivitamin	.963	(.935, .992)	−2.514	.014	1.682	.097
	Placebo	1.000	(.968, 1.033)	−.015	.988		

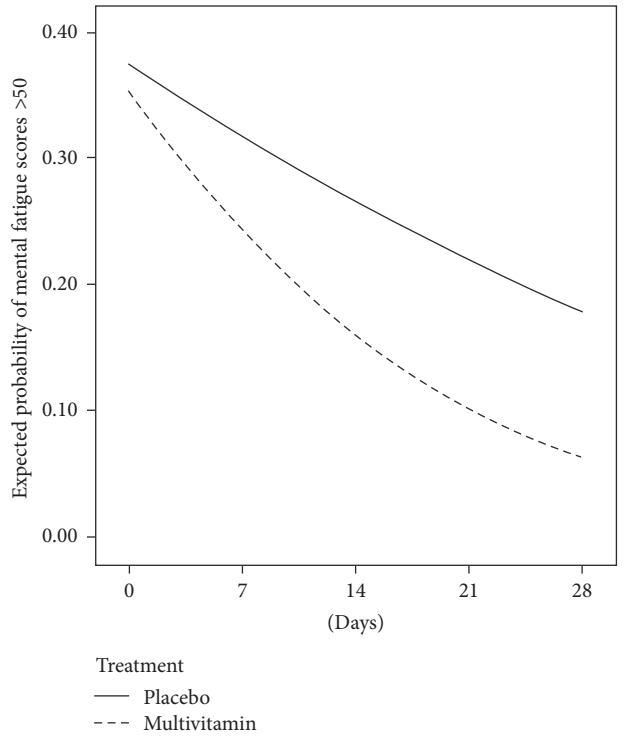

Figure 3: Change in the probability of mental fatigue ratings >50 assessed biweekly using mobile phone devices, commencing at baseline and concluding at the final in-home assessment for someone with 12 years of education.

a period of eight weeks, using a similar MVMH formulation [13]. These results suggest the mood measures used in the present study were suitable to detect mood changes due to the MVMH formula. Furthermore, our analysis from an acute time point in the same study identified immediate mood improvements, particularly improvements to stress, one to two hours after MVMH intake [16]. These findings indicate that the cohort under investigation was responsive to the MVMH treatment and this observation was confirmed in the EMA analysis of mobile phone data undertaken in the current investigation. When considered together the results of this study suggest that the four-week intervention duration may not have been sufficient to induce ongoing mood changes which could be detected in the laboratory when participants had been instructed to abstain from taking the MVMH formula.

There were no multivitamin-related improvements to current mood when measured in the laboratory; however a gradual reduction in stress and mental fatigue was observed in the home across repeated measurements. These results do need to be interpreted with a certain degree of caution as the data was required to be converted into binary form, due to clustering of scores. There are several factors which may have influenced differential home and laboratory results. Firstly, for this cohort the home environment is likely to be less stressful and thus less likely to mask any beneficial mood effects compared to the laboratory. An important consideration is that participants were required to abstain from taking the treatment on the day of the laboratory based follow-up assessments; however at-home biweekly assessments were completed after MVMH intake. Our prior analysis of acute effects of MVMH supplementation from this trial indicated that mood improvements, particularly improvements to stress, occurred 1-2 hours after dose, in the same participant sample, using the same MVMH preparation [16]. Similar immediate mood benefits of multivitamin supplements have been reported by others in younger cohorts [30, 31].

When considered along with previous observations of an acute mood benefit arising from multivitamin supplementation [16], results from the current study suggest that the mood enhancements captured by the EMA methodology may partially reflect more immediate effects of the MVMH formula induced by taking the supplement earlier on the same day as the mood ratings were completed. This interpretation would be consistent with the results of Pipingas et al. [20] who identified benefits to current ratings of mood only on days when participants had consumed the MVMH supplement. Our study extends the findings of Pipingas et al. [20] by demonstrating that multivitamin supplementation can lead to an ongoing reduction in stress and mental fatigue over time and not just when discrete trial end-points are considered. These findings indicate that multivitamin-related benefits to mood may reflect short term postintake mood improvements coupled with a cumulative effect which increases over time. While future studies wanting to solely focus on chronic effects of multivitamins should consider abstinence from treatment prior to posttreatment assessments, the time course and limits of both the acute and cumulative effects of multivitamin supplementation also merit further investigation.

There is evidence from a meta-analysis conducted by Long and Benton [12], which indicates that, across studies, multivitamins significantly improved both stress and mental fatigue facets of mood. This observation has been reported across time frames ranging from hours [16], weeks [28, 29], and through to months after dose [32]. In terms of a putative mechanism, vitamins B2 and B6 and niacin are necessary for amino acid metabolism required for the production of serotonin, a neurotransmitter important for mood regulation [10]. Additionally, vitamins B_{12} and folate and B_6 are crucial for one-carbon metabolism, a process through which *S*-adenosylmethionine (SAMe) is formed. SAMe, the major methyl donor in the body is critical for the production of the neurotransmitters norepinephrine and dopamine, as well as serotonin [33]. Others have shown that three months' supplementation with a drink containing multivitamins and minerals increased levels of serum serotonin, with tryptophan levels increasing over six months [34]. Serum tryptophan levels have been demonstrated to be a useful biomarker to distinguish moderate and severe depression from healthy controls [35], indicating a relationship with mood regulation. Whether changes to levels of serotonin or tryptophan occurred in the present study, especially given the shorter time period, cannot be confirmed. Stress and mental fatigue signify negative aspects of affect, with the former representing a high energy state of activation and the latter a low energy state of deactivation [36]. Greater negative reactivity to stressors has been linked with risk of depression and anxiety in older people [37, 38]; therefore interventions which can reduce the affective impact of psychological stress may contribute to a reduced vulnerability to depression and anxiety. Furthermore, susceptibility to stress has been associated with a greater risk of Alzheimer's disease [39]; therefore reducing stress levels may have implications for cognitive health. Stress and mental fatigue benefits were observed for measures of current mood when measured in the home, rather than retrospective mood ratings, supporting the idea that real-time measures of mood may be more sensitive to the positive effects of nutritional interventions compared to traditional measures [14].

There are several limitations of the study which should be addressed. Mood was rated retrospectively in the laboratory (≥1 week of recall time frame) at the four-week posttreatment visit, when participants were instructed to refrain from taking the MVMH. Future studies need to fully ascertain whether abstaining from MVMH treatment on the day of follow-up assessments attenuates any mood benefits. Differences between mood states when rated in the home setting versus the laboratory also require further investigation. Mood was not rated before dose in the home, as participants were only instructed to rate their mood after consuming the multivitamin. Therefore it is not possible to determine within-day mood changes in the home setting. We utilised the in-lab assessment as our baseline, and it is possible that, due to the setting being the laboratory, mood scores may have been elevated at baseline, relative to whether assessments had taken place in a more naturalistic setting. In contrast to previous RCTs which have examined the mood effects of multivitamin supplements [32, 40], we did not examine any biochemical blood measures of vitamin status. It would have been informative to determine whether levels of B vitamins were sufficient at baseline and whether the MVMH formula was capable of increasing B vitamin levels over the four-week period, given the documented association between folate, vitamin B6, vitamin B12, and mild psychiatric symptoms [4, 12]. The supplement used in the current study also contained a range of 20 botanical extracts. The majority of herbal extracts were at a subtherapeutic dosage; however the MVMH formula contained larger extracts of the herbs Ashwagandha and Ginkgo Biloba which have been implicated in the regulation of mood [41] and flavonoid containing grape seed extract which may exert effects on the central nervous system [42]. The assessment of blood vitamin and flavonoid status at multiple time points corresponding to mood assessments may be useful to provide mechanistic insight into which components of the multivitamin may be responsible for mood alterations.

Findings from this study indicate that mobile phone devices can be used by older women to report on mood in the context of clinical trials designed to evaluate nutritional interventions. On average participants completed 7 of the 8 mood assessments indicating a satisfactory level of compliance in the home setting. In this study only a basic interface was used on the mobile phones allowing for completion of VAS ratings but not more detailed traditional mood measures. The use of a basic interface also led to clustering of scores around similar values, which would not have occurred with a touch screen application, nor on the original paper-and-pencil versions of these measures. Nevertheless due to the number of time points we were still able to identify greater stress reductions for the MVMH group, compared to placebo group, which is consistent with our findings from the acute assessment [16]. The observation of a specific multivitamin-related reduction in stress both acutely [16] and over the longer 4-week period suggests that this reduction in stress

is a valid finding. Similar results of mood improvements detected using EMA measures but not standard pencil and paper measures have been observed following a mindfulness based intervention in emotionally distressed older adults [43]. These results are important as they suggest that EMA approaches could improve the detection of change in patient-reported outcomes in intervention studies when compared to standard paper-and-pencil administration [43]. It is recommended that future studies make use of smart phone applications in order to capture a broader range of mood assessments across a variety of situations (for a review of alternate technology suitable for this purpose see [14]).

In summary, the results of this investigation suggest that, over a period of four weeks, subtle changes to mood, especially stress, may not be detected when only pre- and post-treatment mood are captured. Future research is required to ascertain whether mood improvements due to multivitamin supplementation are driven by an acute effect of consuming the multivitamin [16] and therefore whether mood benefits may be diminished when the treatment is withheld.

Competing Interests

The study was sponsored by Swisse Wellness Pty Ltd. under contract to Swinburne University of Technology and performed independently by the Centre for Human Psychopharmacology. Andrew Pipingas is currently a Member of the Scientific Advisory Panel for Swisse Wellness Pty Ltd.

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Research Article

Improvement of Electroacupuncture on APP/PS1 Transgenic Mice in Spatial Learning and Memory Probably due to Expression of Aβ and LRP1 in Hippocampus

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Objectives. To explore the alterations of β-amyloid (Aβ) and low density lipoprotein receptor-related protein-1 (LRP1) in APP/PS1 mice after electroacupuncture (EA) treatment and further to explore the mechanism. *Methods*. Forty 6-month-old APP/PS1 mice were randomly divided into a model group and an EA group, with twenty wild-type mice used as a normal control group. Mice in the EA group were treated with EA at GV 20 (*bǎi huì*) and bilateral KI 1 (*yǒng quán*) acupoints for 6 weeks. The Morris water maze was applied to assess the spatial memory in behavior. Immunohistochemistry (IHC), ELISA, Western blotting, and so forth were used to observe the expression of LRP1 and Aβ. *Results*. The Morris water maze test showed that, compared with the normal control group, the model group's learning and memory capabilities were significantly decreased ($P < 0.05$; $P < 0.01$). The EA group was reversed ($P < 0.05$; $P < 0.01$). The hippocampal expression of Aβ in the EA group was significantly decreased compared to the model group ($P < 0.01$). The expression of LRP1 in the model group was significantly lower than that in the normal control group ($P < 0.01$); the expression in the EA group was significantly higher than that in the model group ($P < 0.01$). *Conclusions*. EA therapy can improve the learning and memory capabilities of APP/PS1 mice. The underlying mechanism may lie in the upregulation of an Aβ transport receptor and LRP1.

1. Introduction

Alzheimer's disease (AD), the most common cause of dementia in the elderly population, is a kind of neurodegenerative diseases mainly indicated by progressive cognition and memory impairment [1, 2].

In recent decades, the pathological mechanisms of AD have been widely studied [3, 4]. The accumulation of amyloid β-peptide (Aβ) plays the most important role in the pathogenesis of AD [5, 6]. A wealth of evidence has indicated that $A\beta_{1-42}$ deposits participate in the process of neuronal loss which then leads to the occurrence of dementia in AD patients [7]. The high level of Aβ in sporadic AD lies in the imbalance of Aβ production and clearance, especially disordered Aβ clearance [8]. Recent research has focused on Aβ clearance pathways through the cranial microvascular saturable efflux system, namely, transport across the blood-brain barrier (BBB), which has both a fast transport speed and large transport volume. Therefore, the relationship between brain microvessels and the clearance of Aβ is also crucial [9, 10].

A leading hypothesis supports the fact that the main clearing pathway of Aβ in the brain is the transportation of Aβ through the BBB into peripheral blood which has a strong

clearing ability to it [5, 11, 12]. In addition, the BBB model cultured in vitro by the brain microvascular endothelial cell line has also been used to detect whether Aβ can be transported through the BBB, and the finding was affirmative [13, 14]. Aβ is a polar, soluble macromolecular substance [15], and it cannot be freely exchanged between the brain and peripheral blood via free diffusion. Therefore, if Aβ transportation across BBB exists, there must be Aβ specific transporters in the BBB.

Low density lipoprotein receptor-related protein-1 (LRP1) is known to function as a BBB clearance (or efflux) transporter for Aβ. Efflux of Aβ is initiated when it binds directly to LRP1 at the abluminal membrane of the brain endothelial cell [16]. Yamada et al. [17] proved that the brain microvascular endothelial cell uptaking Aβ mainly relies on LRP1 under the BBB-specific cellular context. Bell et al. [18] found in animal experiments that the isotope-labeled Aβ injected into the caudate nucleus of mice would be cleared out from the brain rapidly, and the labeled Aβ was found in the plasma. The clearance of Aβ could be inhibited by LRP1 specific antibodies. Further studies [16, 19, 20] suggest that, in pathological conditions, the abnormality of Aβ levels in the brain might be associated with the altered expression of LRP1 in cranial microvessels.

One survey reported that 55% of patients with AD had tried at least one form of complementary medicine with the hopes that these therapies could improve their overall quality of life and delay further decline in cognitive functioning. Clinical research has shown that acupuncture can improve the mental and behavioral conditions of AD patients, as well as the cognitive function [21–23]. Electroacupuncture (EA) is a simple and effective modern acupuncture method used in the treatment of many diseases. A previous study has reported that EA at GV 20 (*bǎi huì*) shows a significant protective effect on neuronal damage and impairment of learning and memory [24]. More evidences have proven that acupuncture has a therapeutic effect on AD [25–27]. However, the mechanism is still unclear and more exploration is needed.

In this study, 6-month-old APP/PS1 transgenic mice were selected as the animal model of AD. EA at GV 20 (*bǎi huì*) and KI 1 (*yǒng quán*) was given to the mice. The effects on their behavior and the expression of $A\beta_{1-42}$ and LRP1 levels in the hippocampus were observed and analyzed so as to explore the treatment mechanism of EA on early intervention of AD.

2. Materials

2.1. Animal Model and Grouping

2.1.1. Animals. 6-month-old APPswe/PS1dE9 double transgenic male mice were used as the animal model of AD, and wild-type mice with the same age and sex were used as the normal control group. Animals were purchased from Model Animal Research Center of Nanjing University (animal lot: SCXK (Ning) 2010-000), weighing 34.2 ± 3.98 g. All experimental procedures comply with the guidelines of the "Principles of Laboratory Animal Care" formulated by the National Institute of Health and the legislation of the People's Republic of China for the use and care of laboratory animals. The experimental protocols were approved by the Animal Experimentation Ethics Committee of Beijing University of Chinese Medicine. Efforts were made to minimize the number of animal uses and the suffering of the experimental animals.

2.1.2. Animal Grouping and Intervention. 40 APP/PS1 transgenic mice were randomly divided into two groups, a model group (M) ($n = 20$) and an electroacupuncture group (EA) ($n = 20$), while 20 wild-type mice were selected as the normal control group (C). All mice were raised with a regular diet in single cages in the barrier system of the Animal Center of Beijing University of Chinese Medicine.

Regarding EA group, EA on GV 20 (*bǎi huì*) and bilateral KI 1 (*yǒng quán*) was given to the mice, with transversely puncturing at a depth of 2-3 mm by disposable sterile acupuncture needles (0.25 mm × 13 mm) (Beijing Zhongyan Taihe Medicine Company, Ltd.). The anode and cathode of EA were, respectively, connected to the left and right KI 1 (*yǒng quán*), with a dilatational wave at a frequency of 2/15 Hz, 1 mA, and an intensity that the needle tremors, while animals keep quiet by Han's acupoint nerve stimulator (Beijing Huawei Industrial Development Company, Han's LH202H type). GV 20 (*bǎi huì*) is located at the intersection of the sagittal midline and the line linking two rat ears [26]. KI 1 (*yǒng quán*) is located on the sole of the foot, at the indentation near the front part, between the second and third metatarsal bones, 1/3 of the distance from the webs of the toes to the heel [28]. The EA treatment was performed with mice restrained in mouse bags, 15 mins per time, once every other day for 6 weeks. Mice in the normal control group and the model group were just restrained in the same mouse bags for 15 min, once every other day, totally for six weeks [29].

3. Methods

3.1. Learning and Memory Behavioral Testing. Morris water maze testing was conducted in a round pool to test the behavior of learning and memory [30]. Water maze training was given on the 2nd–6th days after EA intervention for 6 weeks, with water temperature at 22 ± 2°C. One day before the experiment, mice in each group were placed on the platform for 10 s to adapt to the environment, and each mouse was offered a period of free swimming in the maze for 1 min with the fourth quadrant as the entry point. On the first to fourth days of the experiment, mice in each group were given a place navigation test. Mice were firstly placed on the platform to adapt to the environment for 10 s and then were sequentially put into water facing the wall away from the four quadrants in sequence. The time recording was stopped after 5 s of the mouse on the platform, and the maximum swim time was set at 60 s. On the fifth day, the spatial probe test was performed. The platform was removed, and mice were sequentially placed directly into the water from the four quadrants. A computer connected to an image analyzer (BS-124S Morris water maze video analysis system: provided by the Pharmacology Laboratory of Dongzhimen Hospital affiliated to Beijing University of Chinese Medicine, Shanghai

Mobile Information Technology Co., Ltd.) monitored the swim pattern.

3.2. Collection of Brain Tissues and Detection of Related Indexes

3.2.1. $A\beta_{1-42}$ Immunohistochemistry

Sample Preparation. The brains of 2 mice in each group were fixed in paraformaldehyde after cardiac perfusion and then trimmed, dehydrated with ethanol, made transparent with xylene, embedded in paraffin, and sectioned on a coronal plane.

$A\beta_{1-42}$ Immunohistochemical ABC Method. Sections were firstly dewaxed and hydrated and were put into 0.01 mol/L citrate buffer for antigen thermal remediation for 10 min and 3% methanol hydrogen peroxide at room temperature for 10 min. Then the sections were blocked in 5% normal goat serum at 37°C for 30 min and incubated with primary antibody diluent (USA, Abcam, ab10148, 1 : 100) for one night at 4°C. After incubation within the primary antibody diluent, the sections were rinsed with phosphate-buffered saline (PBS) and then incubated with secondary antibody diluent (Boster Biological Engineering, goat anti-rabbit HRP-IgG, 1 : 1000) for 90 minutes at 37°C. The sections were rinsed with PBS before incubation with AB complex for 90 minutes at 37°C and were placed into diaminobenzidine (DAB) solution for 10 minutes after being rinsed another time with PBS. After being redyed with hematoxylin, they were dehydrated and mounted after transparence. The sections were observed under the microscope (BX53, Olympus Corporation, Japan).

3.2.2. Laser Confocal Imaging of Frozen Section Observation with Laser Scanning Confocal Microscope

Sample Preparation. With 3 mice in each group, the analytes were fixed in paraformaldehyde after cardiac perfusion. 30% sucrose was then added after 24 hours, and brain tissues were frozen sectioned with OTC embedded when sunk into the bottom of the bottle.

Proteinase K was added at 37°C after the brain tissues were washed three times with PBS with 5 mins each. 30 min later, LRP1 (USA, Abcam, ab28320, 1 : 70) and $A\beta_{1-42}$ (USA, Abcam, ab10148, 1 : 100) were added equivalently after washing for three times with PBS as above. With incubation overnight at 4°C, the second antibodies (FITC (USA, Abcam, ab6785, 1 : 250) and Alexa Fluor® 647 (USA, Abcam, ab150079, 1 : 150)) were added after washing with the same method. With incubation for 1.5 hours at indoor temperature, DAPI (Beijing Zhongshan Golden Bridge, ZLI-9557) was added to fix the sections after washing as before. A laser scanning confocal microscope (FV1000, Olympus Corporation, Japan) was used for sections observation.

3.3. Collection and Preservation of Brain Tissues. After the water maze test, mice in each group were anesthetized with 0.3% sodium pentobarbital (30 mg/kg). The 15 hippocampi were taken with craniotomy and prepared for ELISA and Western blotting samples in time on the left and right sides, respectively, and then preserved in a refrigerator at 80°C.

3.4. Preparation of Samples

3.4.1. For $A\beta$ ELISA. The 6 right hippocampi were weighed in each group and homogenized with 8 times the volume of mixed liquor of 5 M guanidine hydrochloride, 50 mM Tris hydrochloric acid (pH 8.0), and 1 mM PMSF on ice. Then the hippocampi were centrifuged with 16000 r/min, at 4°C, for 20 min, and the supernatant was obtained. The diluted samples were prepared by separately mixing the supernatant with a standard diluent of 3200 times the volume (KHB3441, Invitrogen, USA) and 800 times the volume (KHB3441, Invitrogen, USA) with the hippocampus of the model and EA groups. The samples in the normal control group were not diluted.

3.4.2. For LRP1 Western Blotting. The 6 left hippocampi were added to the RIPA lysate solution containing 1 mM PMSF with the weight ratio of 1 : 100 in each group, and then the total protein was extracted from tissues after homogenizing. Then the hippocampi were centrifuged at 2000 r/min, at 4°C, for 10 minutes, after extraction, and the volume of the supernatant was calculated. The samples were then prepared for Western blotting.

3.4.3. For LRP1 ELISA. The remaining 9 hippocampi were weighed in each group and homogenized with a dilution of 1 : 8 in PBS. The supernatant was extracted with the same extracting method of 6 right hippocampi. The diluted samples to be tested were prepared by adding the supernatant with a standard dilution of 1 : 5 (DRE20100, RD, USA) in all groups.

3.5. Double Antibody Sandwich Method for ELISA. The ELISA kit was used to, respectively, detect samples. First, 50 μL of antibody operating solution was added into each plate at room temperature for 120 minutes. Then 100 μL horseradish peroxidase labeled secondary antibody operating solution was added to each plate at room temperature and dark reaction for 30 minutes. Complete plate washing was performed after four attempts. A 100 μL chromogenic substrate operating solution was added to each plate at room temperature and dark reaction for 30 minutes. Plate washing was performed after four completed attempts. Then 100 μL stop solution was added and mixed into each plate. The absorbance was detected at the 450 nm place with a microplate reader within 30 minutes. The standard protein line was drawn with Excel. The sample concentration was converted according to the sample readings, and $A\beta_{1-42}$, $A\beta_{1-40}$, and LRP1 concentrations were calculated according to the sample diluted concentration.

3.6. Western Blotting. SDS-PAGE electrophoresis was performed with a 10% separating gel and a 5% stacking gel and transferred to a 0.45 μm PVDF membrane. Membrane blocking was performed using 5% nonfat milk in Tris buffered saline supplemented with 0.1% Tween 20 (TBST). The first antibody (USA, Abcam, ab92544, 1 : 20000; ab8227, 1 : 2000)

Table 1: Comparison of escape latency time in each group in Morris water maze place navigation test ($\overline{x} \pm s$, s, $n = 20$).

Groups	Cases	Day 1	Day 2	Day 3	Day 4
Normal control group (C)	20	54.19 ± 6.13	45.96 ± 11.64$^{\blacktriangle\blacktriangle}$	40.33 ± 14.89$^{\blacktriangle\blacktriangle}$	37.93 ± 13.78$^{\blacktriangle\blacktriangle}$
Model group (M)	20	58.32 ± 4.6$^{\divideontimes\divideontimes}$	60.00 ± 0.00$^{\divideontimes\divideontimes}$	55.39 ± 8.30$^{\divideontimes\divideontimes}$	54.65 ± 9.87$^{\divideontimes\divideontimes}$
EA group (EA)	20	58.14 ± 5.07	57.05 ± 5.72**	52.45 ± 9.40$^{\blacktriangle\blacktriangle}$	48.65 ± 12.71$^{*\blacktriangle\blacktriangle}$

Notes: $^{\divideontimes\divideontimes}$compared with the normal control group, $P < 0.01$; *compared with the model group, $P < 0.05$; **compared with the model group, $P < 0.01$; $^{\blacktriangle\blacktriangle}$compared with the first day of the same group, $P < 0.01$.

Table 2: Comparison of platform crossover number and swimming distance in platform quadrant of each group in Morris water maze spatial probe test ($\overline{x} \pm s$, time, cm, $n = 20$).

Groups	Cases	Platform crossover number	Swimming distance in platform quadrant
Normal control group (C)	20	1.74 ± 1.4	367.35 ± 142.89
Model group (M)	20	0.50 ± 0.44$^{\divideontimes\divideontimes}$	192.46 ± 72.00$^{\divideontimes\divideontimes}$
EA group (EA)	20	0.90 ± 0.71*	296.61 ± 105.84**

Notes: $^{\divideontimes\divideontimes}$compared with the normal control group, $P < 0.01$; *compared with the model group, $P < 0.05$; **compared with the model group, $P < 0.05$.

was added prior to incubation for one night at 4°C. The secondary antibody (USA, Abcam, ab6721, 1 : 2000) was added before shaking and incubating at room temperature for 1.5 h. HRP-ECL luminous liquid was added and the X-ray film exposure was completed in a dark room following the developing and fixing. After calibrating the markers, analysis and scanning were performed, and the relative expression of LRP1 (LRP1/β-actin gray value) was compared in each group.

4. Statistical Analysis

SPSS 17.0 software was used to conduct the statistical analysis. All data were presented as means ± standard deviation ($\overline{x} \pm s$). Variance analysis of multigroup repeated measurement design date was adopted for the data of the Morris water maze behavioral escape latency. One-way ANOVA was used after the test of normal distribution and homogeneity of variance, and LSD method was used for pairwise comparisons for the ELISA detection and Western blotting. If there was a non-normal distribution or heterogeneity of variance for the data, a nonparametric test would be used. Statistical significance was set to $P < 0.05$, while highly statistical significance was set to $P < 0.01$.

5. Results

5.1. Effect of EA on Spatial Learning and Memory. Statistical results showed that, according to variance analysis of repeated measurement and effect between groups, the escape latency time in each group was significantly decreased with the increase of training days, and there were significant differences among groups ($P < 0.05$). There was a significant difference for the training time (day) ($P < 0.05$), but there was no significant difference for the interaction between the training time and groups (day × group) ($P > 0.05$). Pairwise comparison results showed that the escape latency time in the model group was significantly longer than that in the control and EA groups (see Table 1 and Figure 1).

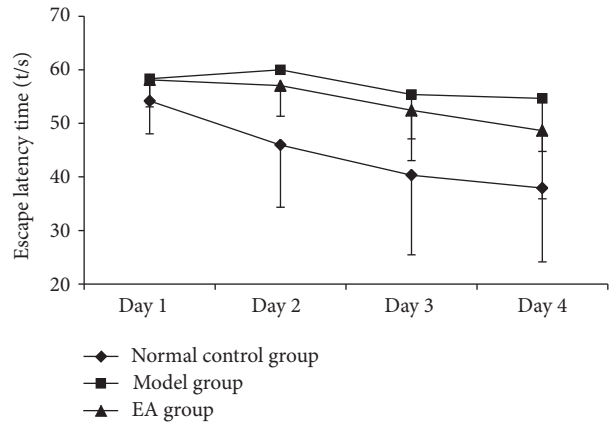


Figure 1: Comparison of escape latency time in each group in Morris water maze place navigation test.

One-way ANOVA was used to determine the number of platform crossovers and the swimming distance in the platform quadrant for the spatial probe test for the three groups, and the differences were statistically significant ($P < 0.05$). The number of platform crossovers and the swimming distance in the platform quadrant of the model group were significantly lower than those in the normal control group ($P < 0.01$). Compared with the model group, the number of platform crossovers of the EA group was significantly higher ($P < 0.05$), and the swimming distance in the platform quadrant in the EA group was significantly longer ($P < 0.01$) (see Table 2).

The swimming trajectories of mice in the normal control group were mostly concentrated in the original target platform quadrant, and the subjects tended to search mainly. However, the type of searching in the model group was random. Compared to the model group, the swimming trajectories of mice in the EA group were more concentrated in the original target quadrant or adjacent quadrants, and the

Table 3: Effect of EA on $A\beta_{1-42}$ and $A\beta_{1-40}$ expression ($\overline{x} \pm s$, pg/mg, $n = 6$).

Groups	Cases	$A\beta_{1-42}$	$A\beta_{1-40}$	$A\beta_{1-42}/A\beta_{1-40}$
Normal control group (C)	6	0.14 ± 0.38	0.077 ± 0.01	1.79 ± 0.61
Model group (M)	6	$6119.76 \pm 670.13^{\text{※※}}$	$801.05 \pm 219.24^{\text{※※}}$	$7.97 \pm 1.61^{\text{※※}}$
EA group (EA)	6	$1326.58 \pm 501.40^{**}$	$297.05 \pm 112.89^{**}$	7.02 ± 1.73

Notes: ※※compared with the normal control group, $P < 0.01$; **compared with the model group, $P < 0.01$.

Table 4: Effect of EA on relative expression level of LRP1 ($\overline{x} \pm s$, $n = 6$).

Groups	Cases	LRP1 gray value ($\times 10^4$)	Actin gray value ($\times 10^4$)	LRP1/Actin
Normal control group (C)	6	6.77 ± 1.17	11.16 ± 0.86	0.60 ± 0.08
Model group (M)	6	$3.87 \pm 0.76^{\text{※※}}$	11.77 ± 1.24	$0.33 \pm 0.07^{\text{※※}}$
EA group (EA)	6	$6.34 \pm 1.32^{**}$	11.86 ± 0.74	$0.53 \pm 0.10^{**}$

Notes: ※※compared with the normal control group, $P < 0.01$; **compared with the model group, $P < 0.01$.

searching trend had a linear and trending appearance (see Figure 2).

5.2. Effect of EA on Aβ and LRP1

5.2.1. Immunohistochemistry Results of $A\beta_{1-42}$ in Hippocampus. In the normal control group, there were brown positive expressions of $A\beta_{1-42}$ inside the cell and negative ones outside the cell. In the model group, there were positive expressions of $A\beta_{1-42}$ and plaque deposits with compactness outside the cell. Compared with the model group, the expression of $A\beta_{1-42}$ in EA group was significantly weakened, and there were a few diffuse senile plaques (see Figure 3).

5.2.2. Laser Confocal Imaging of $A\beta_{1-42}$ and LRP1 in Hippocampus. In the normal control group, plaque deposits of $A\beta_{1-42}$ were not found, while LRP1 was expressed mostly around the vascular endothelial cells. In the model group, there were dense-core plaques deposited, while less LRP1 was expressed. In EA group, the senile plaques were relatively reduced, and there were only some diffuse plaques, while the expression of LRP1 was more than that of model group. $A\beta_{1-42}$, LRP1, and cell nucleus were labeled with laser confocal imaging (see Figure 4).

5.2.3. Effect of EA on $A\beta_{1-40}$ and $A\beta_{1-42}$ Expression. The ELISA results were shown in Table 3. LSD test was used for pairwise comparison. Results showed that $A\beta_{1-42}$ and $A\beta_{1-40}$ expression levels and $A\beta_{1-42}/A\beta_{1-40}$ values in the model group were all higher than those of the normal control group ($P < 0.01$); $A\beta_{1-42}$ and $A\beta_{1-40}$ expression levels in the EA group were lower than those in the model group ($P < 0.01$), while there was no significant difference for $A\beta_{1-42}/A\beta_{1-40}$ between the EA and model groups.

5.2.4. Effect of EA on Relative Expression Level of LRP1. Western blotting results of LRP1 were shown in Table 4 and Figure 5. The LRP1/β-actin gray value was the relative expression level of LRP1.

LRP1/Actin. There was a significant difference between the model group and the normal control group ($P < 0.01$), and the EA group was significantly higher than the model group ($P < 0.01$) (see Table 4).

Table 5: Effect of EA on LRP1 expression ($\overline{x} \pm s$, pg/mg, $n = 9$).

Groups	Cases	LRP1
Normal control group (C)	9	0.21 ± 0.25
Model group (M)	9	$0.17 \pm 0.1^{\text{※※}}$
EA group (EA)	9	0.19 ± 0.11

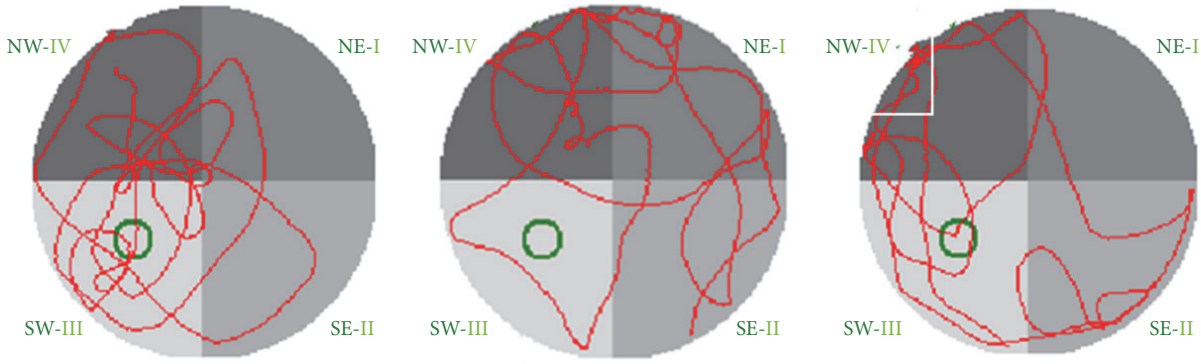
Notes: ※※compared with the normal control group, $P < 0.01$.

Table 5 shows the ELISA test results of LRP1 expressions. There was a significant difference between the model group and the normal control group ($P < 0.01$). There was no significant difference between the EA group and the model group ($P > 0.05$), but the EA group was higher than the model group.

6. Discussions

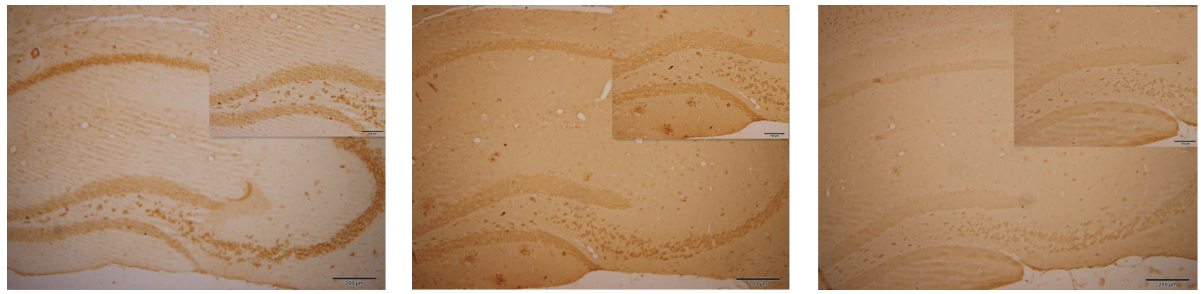
The disordered clearance of Aβ is the main pathogenesis of AD. Aβ is cleared out through the BBB or degraded through the pathway of brain cell fluid drainage [31, 32]. The Aβ level between the brain parenchyma and brain interstitial fluid is inversely proportional to the age of mice. For the older mice, the Aβ level of brain parenchyma increases, while the Aβ level of brain interstitial fluid decreases [33]. The experimental study has found that LRP1 can transport Aβ out from BBB to be involved in the clearance of Aβ vessels [34], and the Aβ level can be decreased by transporting it.

In 1997, Narita et al. [35] discovered that there was a correlation between LRP1 and AD. However, studies on the pathogenesis of AD have shown that the functions of LRP1 are complicated, and some results are contradictory. On one hand, LRP1 can internalize APP and decompose it as Aβ in organelle lumen; on the other hand, LRP1 on neuronal membrane can also perform enzymatic degradation through the endocytosis of Aβ with its ligand α2M and ApoE; meanwhile, LRP1 on brain microvascular endothelial cells may mediate the outflow transport of Aβ across the BBB. Therefore, LRP1 is correlated to the generation and clearance of Aβ in some degree [36–38]. Studies have indicated that, for the AD model, the transportation of Aβ across the BBB



(a) Normal control group (C) (b) Model group (M) (c) EA group (EA)

Figure 2: Swimming trajectories with fourth quadrant as water entry point in each group in Morris water maze spatial probe test: (a) normal control group: the swimming trajectory was mostly concentrated in the original target platform quadrant; (b) model group: the swimming trajectory was random. (c) EA group: the swimming trajectories were concentrated in the original target quadrant or adjacent quadrants.



(a) Normal control group (C) (b) Model group (M) (c) EA group (EA)

Figure 3: Immunohistochemistry of $A\beta_{1-42}$: complete view (original magnification ×100, 1 : 200); sectional view (original magnification ×200, 1 : 100); (a) normal control group: there were brown positive expressions of $A\beta_{1-42}$ inside the cell but negative outside the cell; (b) model group: there were positive expressions of $A\beta_{1-42}$ and plaque deposits with compactness outside the cell; (c) EA group: there were a few diffuse senile plaques.

mediated by LRP1 in the brain is damaged [39, 40]. The inhibition of LRP1 expression in brain of healthy mice could decrease the outflow rate of $A\beta$ in brain by 30% [41] and prompt the sedimentation of $A\beta$ in brain.

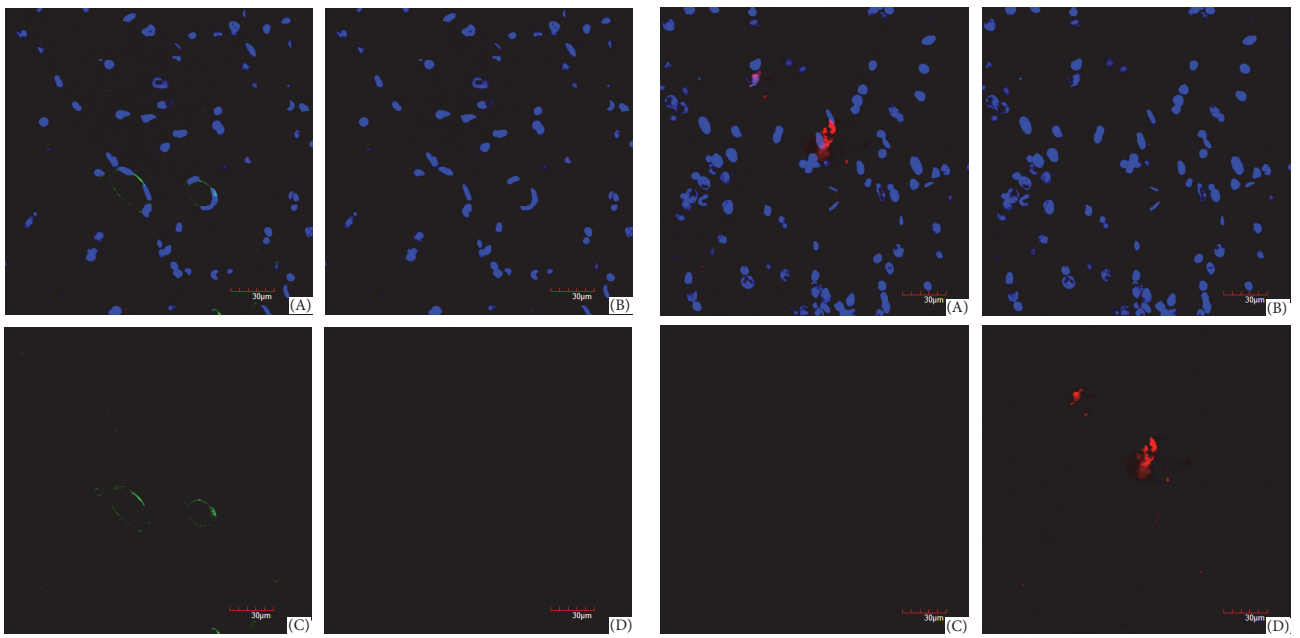
Studies have shown that, by injecting iodine-labeled $A\beta$ into the brain, the rapid transport process of $A\beta$ across the BBB can be observed [42]. This process can be inhibited by LRP1's sensitive inhibitor (RAP) and $\alpha 2$ macroglobulin. All these evidences prove that LRP1 on the BBB may be the main carrier of $A\beta$ transported out from the brain; that is, LRP1 is involved in the clearance of $A\beta$, and it also has a negligible effect on the prevention of AD symptoms.

Experiments have shown that electroacupuncture can improve learning and memory abilities in transgenic mice and decrease the $A\beta$ level in brain. The mechanism may be related to the effect of electricity on the cerebral microvessels and the $A\beta$ transport receptor LRP1 [13, 18, 43].

Our results show that, in the water maze escape latency, mice in the normal control group and the EA group had separately retained the spatial memory in the 2nd and 3rd days, and the escape latency time shortened with the increase in days. It indicates that EA has improved the AD models' ability of spatial learning and memory. ELISA testing displayed that the levels of $A\beta$ were significantly decreased, which shows a consistency with the results of immunohistochemistry and laser confocal imaging. This may be a mechanism of EA's improvement of the memory of AD mice.

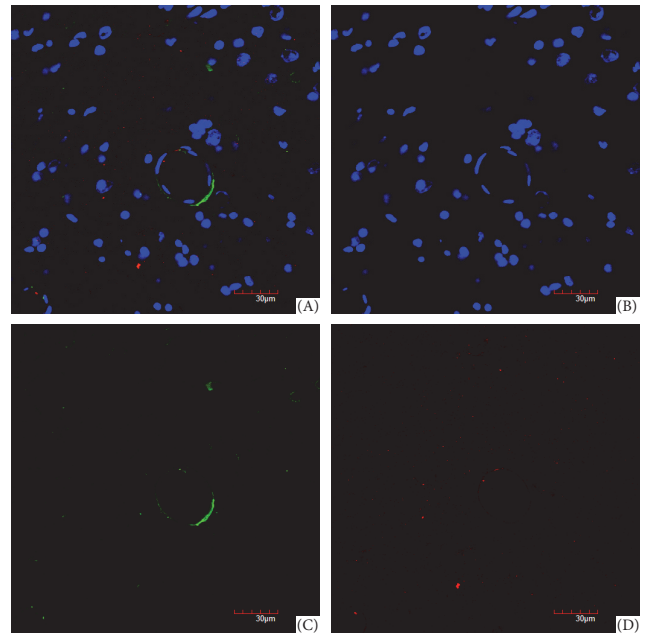
The AD animal models are APPswe/PS1dE9 double transgenic mice prepared by transferring the human mutated genes APP and PS1 into mice to raise the $A\beta$ levels in brain so as to cause a series of pathological lesions of AD. This model assumes that $A\beta$ is the main pathological pathogenic factor. It was reported previously that the escape latency time in spatial probe test increased for 3-month-old APPswe/PS1dE9 double transgenic mice [44], while in the preliminary study of this experiment, it was found that the Morris water maze escape latency time in EA group and model group was not significantly different from that in normal control group for 5-month-old double transgenic mice. The water maze experiments of Li et al. [26] have indicated that the learning and memory disorders begin to appear at the age of 7-8 months.

This study has shown that EA can improve the spatial learning and memory of 7-month-old APP/PS1 transgenic mice in the model group which have been shown to have both learning and memory disorders.



(a) Normal control group (C)

(b) Model group (M)



(c) EA group (EA)

FIGURE 4: Laser confocal imaging of $A\beta_{1-42}$ and LRP1 in hippocampus (original magnification ×600): (A) stack imaging of the three; (B) imaging of cell nucleus; (C) LRP1 labeled with green fluorescence; (D) $A\beta_{1-42}$ labeled with red fluorescence; (a) in normal control group, plaque deposits of $A\beta_{1-42}$ were not found, while LRP1 was expressed mostly around the vascular endothelial cells; (b) in model group, there were dense-core plaques deposited, while less LRP1 was expressed; (c) in EA group, the senile plaques were relatively reduced, and there were only some diffuse plaques, while the expression of LRP1 was more than that of model group.

Our research group has previously done a study of EA on GV 20 (*bǎi huì*) and KI 1 (*yǒng quán*) as the intervention to APPV717I AD mice. EA could intervene in the behavior of 11-month-old APP single transgenic AD mice, neuronal changes, and Aβ protein expression in brain, especially where there is a decreasing trend of Aβ sedimentation in the hippocampus microvessels [43]. EA therapy can regulate the APP/PS1 double transgenic mice hippocampus ultrastructure [45]. APP/PS1 double transgenic mice were used in this study which could cause earlier pathological manifestations such as Aβ sedimentation, senile plaques, neuronal loss, and behavioral obstacles of cognitive performance compared

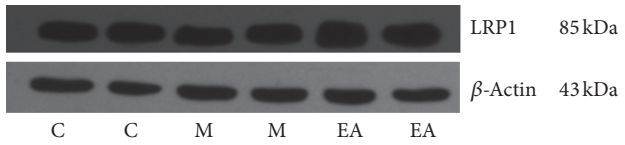


Figure 5: Effect of EA on relative expression level of LRP1.

to single-transgenic mice, so that the length of the EA experiment cycle can be shortened. The appearance of senile plaques on brain cortex for 7-month-old APP/PS1 transgenic mice has indicated an early intervention of EA.

The focus of this study is on the effect of EA on LRP1, which is the key receptor in Aβ clearance of vessels in brains of the models. LRP1 mainly exists in cerebral vascular endothelial cells, vascular smooth muscle cells, and other cells including neurons, astrocytes, and smooth muscle cells [46]. In this study, the laser scanning confocal microscope was applied to observe the coexpression of LRP1 and Aβ in the hippocampus, proving that LRP1 is expressed on the microvascular endothelial cells.

This experiment showed that the Aβ levels in the model group increased, while EA can decrease Aβ levels. Furthermore, LRP1 levels in the model group decreased, while EA increased LRP1 levels. Therefore, the mechanism of EA's involvement in improving learning and memory may be its ability to increase LRP1 levels, thus increasing the clearance of Aβ.

It has been reported that Aβ can be directly transported by LRP1, and studies have also shown that Aβ can be only transported after forming complexes with other ligands of LRP1 such as ApoE [47]. Does EA regulate another mechanism of ApoE and so forth by increasing the rate and quantity of combination of Aβ and LRP1 and further decrease Aβ? Further studies of possible mechanisms are required.

In summary, this study has indicated that EA can improve learning and memory of mice. Aβ levels were shown to be significantly lower in the EA group compared to the model group, while LRP1 levels were also significantly higher in the EA group, which may indicate that the decreasing of Aβ is related to the transportation of LRP1; therefore EA may be involved in regulating LRP1 functions. Enhancing the transportation of Aβ protein and decreasing the Aβ levels may be a functional way to treat dementia using EA, but still further evidence is needed.

7. Conclusions

EA therapy can improve both learning and memory capabilities in APP/PS1 transgenic mice. The underlying mechanism may be due to the upregulation of the Aβ transport receptor LRP1, thus acting on learning and memory by contributing to decreased levels of Aβ in the hippocampus.

Ethical Approval

All procedures performed in studies involving animals were in accordance with the "Principles of Laboratory Animal Care" formulated by the National Institute of Health and the legislation of the People's Republic of China for the use and care of laboratory animals.

Consent

Informed consent was obtained from all individual participants included in the study.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Xin Wang and Yanhuan Miao contributed equally to this work.

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Research Article

Electroacupuncture Ameliorates Learning and Memory and Improves Synaptic Plasticity via Activation of the PKA/CREB Signaling Pathway in Cerebral Hypoperfusion

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Electroacupuncture (EA) has shown protective effects on cognitive decline. However, the underlying molecular mechanisms are ill-understood. The present study was undertaken to determine whether the cognitive function was ameliorated in cerebral hypoperfusion rats following EA and to investigate the role of PKA/CREB pathway. We used a rat 2-vessel occlusion (2VO) model and delivered EA at Baihui (GV20) and Dazhui (GV14) acupoints. Morris water maze (MWM) task, electrophysiological recording, Golgi silver stain, Nissl stain, Western blot, and real-time PCR were employed. EA significantly (1) ameliorated the spatial learning and memory deficits, (2) alleviated long-term potentiation (LTP) impairment and the reduction of dendritic spine density, (3) suppressed the decline of phospho-CREB (pCREB) protein, brain-derived neurotrophic factor (BDNF) protein, and microRNA132 (miR132), and (4) reduced the increase of p250GAP protein of 2VO rats. These changes were partially blocked by a selective protein kinase A (PKA) inhibitor, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline-sulfonamide (H89), suggesting that the PKA/CREB pathway is potentially involved in the effects of EA. Moreover, any significant damage to the pyramidal cell layer of CA1 subregion was absent. These results demonstrated that EA could ameliorate learning and memory deficits and alleviate hippocampal synaptic plasticity impairment of cerebral hypoperfusion rats, potentially mediated by PKA/CREB signaling pathway.

1. Introduction

Due to an increase in aging population, age-related cognitive impairment, in particular vascular cognitive impairment (VCI), becomes increasingly challenging worldwide, without effective medications [1]. As a novel combinational approach, electroacupuncture (EA) therapy, consisting of traditional acupuncture and modern electrotherapy technology, was frequently reported to alleviate cognitive decline in patients with stroke [2], Alzheimer disease (AD) [3, 4], or mild cognitive impairment (MCI) [5–7]. In addition, EA was suggested to prevent cognitive deficits in rats with cerebral ischemia [8–11]. However, the underlying molecular mechanisms are not entirely understood.

cAMP response element-binding protein (CREB)/CRE-mediated gene transcription and protein synthesis have been speculated to be essential in long-term hippocampal synaptic plasticity and memory formation [12–16]. Several pieces of evidence have confirmed that protein kinase A (PKA) is one of the major kinases to activate CREB and the PKA/CREB pathway is critical in learning and memory [17]. Previous studies had revealed that transcription of

BDNF gene was CREB-regulated in an activity-dependent manner, wherein its expression was involved in neuronal development, synaptic plasticity, and neuroprotection [13]. For example, BDNF was related to the selective vulnerability of hippocampal neuronal populations during brain injury [18]. It also played a vital role in long-term potentiation (LTP), a cellular model for learning and memory [19, 20]. Of note, recently, miRNA132 (miR132) was identified as a novel CREB target gene and was suggested to regulate neuronal morphogenesis through translational inhibition of a GTPase-activating protein, p250GAP [21].

Hitherto, only a few studies about the molecular alterations of PKA/CREB pathway during EA treatment have been reported. In agreement, acupuncture treatment at Zusanli (ST36) was shown to increase cAMP concentration, PKA activity, pCREB protein, and pERK protein expressions, accompanied by alleviation of memory impairment in cerebral multi-infarction rats [8]. In addition, one of our previous studies had shown that EA attenuated neuronal apoptosis and ameliorated learning and memory in 2VO rats, via increasing the expressions of pCREB and BCL-2 protein [22].

Based on the above findings, we hypothesized that treatment of EA might enhance hippocampal synaptic plasticity and improve cognitive function of cerebral ischemic rats and that PKA/CREB signaling pathway might be a potential mechanism underlying these effects. Firstly, spatial learning and memory, LTP and dendritic spine density, and neuronal viability as well as expression of pCREB, BDNF, miR132, and p250GAP proteins were examined to test effects of EA in cerebral hypoperfusion. Furthermore, to test the hypothesis that the PKA/CREB pathway is involved in those beneficial effects of EA, rats were pregiven a selective PKA inhibitor, H89 (it can strongly inhibit the biological action of PKA), and similarly monitored for the above-mentioned indicators. Thus, the present study was aimed at determining whether and how the cognitive function and the hippocampal synaptic plasticity were altered by EA in cerebral hypoperfusion. Also, we investigated the probable underlying molecular machinery comprised of the PKA/CREB pathway.

2. Materials and Methods

2.1. Animals. A total of 87 adult male Sprague-Dawley rats weighing between 250 g and 300 g were used (SPF grade, Experimental Animal Center, Huazhong University of Science and Technology, Wuhan, China). Animals were housed in a group of 4-5/cage at 24 ± 1°C in a light-controlled (12 h light/dark cycle) room, providing free access to water and food. All the animal studies were approved by the Review Committee for the Care and Use of Laboratory Animals of Tongji Medical College, Huazhong University of Science and Technology.

2.2. Establishment of 2VO Model. Animals were randomly assigned into five groups: (1) control group: rats were anesthetized and exposed to surgery but did not deal with actual ligation; (2) model group: rats have undergone 2VO operation; (3) EA group: rats were given EA treatment after 2VO operation, for seven consecutive days; (4) EA+H89 group: rats were given H89 at 30 min before the operation of 2VO and then received EA similarly to the EA group; and (5) EA+normal saline (NS) solution group: rats were injected with equal volume of NS instead of H89, at 30 min before the 2VO operation and the subsequent EA treatment.

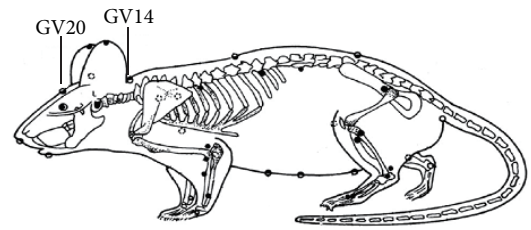

Figure 1: Rat schematic showing the location of the acupuncture points used in the study. GV14 represents "Dazhui," which is located on the posterior midline and in the depression below the spinous process of the seventh cervical vertebra; GV20 represents "Baihui," which is located at the right midpoint of the parietal bone.

H89 (2 μg/μL × 10 μL, Sigma-Aldrich, Shanghai, China) or a vehicle of sterile NS was intracerebroventricularly (icv) injected into rats of EA+H89 group or EA+NS group. Under intraperitoneal anesthesia (10% chloral hydrate, 350 mg/kg, intraperitoneally (ip)), the head was mounted in a stereotaxic instrument (SN-3, Narishige, Japan). Injection coordinates were 0.8 mm posteriorly to the bregma, 1.5 mm laterally to the midline in the right hemisphere, and 4.5 mm below the dural surface.

Through a ventral midline cervical incision, both common carotid arteries were carefully isolated from their sheaths and vagal nerves and doubly ligated with 4-0 silk thread. After occlusion, most animals appeared normal, but few rats that displayed epileptic seizures (2/87) or absence of weight gain (3/87) or intestinal obstruction (2/87) were excluded from subsequent experiments.

2.3. EA Treatment. EA was commenced one day after the operation in conscious rats. The location of "Baihui" acupoint (GV20) and "Dazhui" acupoint (GV14) was described previously [23] (Figure 1).

> *"Acupuncture needles were inserted into the muscle at a depth of 0.5 mm of GV20 to serve as a cathode while another electrode was placed on the GV14 serving as an anode. Then electrical stimulation was delivered using a G6805-II electroacupuncture therapeutic apparatus (Shanghai Medical Electronic Apparatus Co., China), with continuous current at 20 Hz for 20 min daily. Stimulus intensity was based on the visible light facial muscles twitching."*

2.4. Morris Water Maze Task. Morris water maze (MWM) task was carried out to test spatial learning and memory. Briefly, a circular water tank (150 cm in diameter and 50 cm deep) was filled with 23 ± 2°C water to a depth of 21 cm. A circular platform of 15 cm in diameter and 20 cm height

was placed in the center of the target quadrant (quadrant I). Several visual cues were located on the wall of the test room. Rats were subjected to two sessions of four place navigation trials per day with an interval of at least 4 h for three consecutive days (day 5–day 7, training for 5 times). The starting points were changed for every trial. On day 8, the platform was removed for spatial probe trial to test the spatial memory. The latency to find the submerged platform and the dwell time in quadrant I and the swimming paths were recorded automatically using a computer-based image analyzer MWM tracking system MT-200 (ChengDu Technology & Market Co., Ltd., Chengdu, Sichuan Province, China).

2.5. Electrophysiological Recording. Rats were anesthetized with urethane (1.5 g/kg, ip) and placed in a stereotaxic frame (SN-3, Narishige, Japan) for surgery and recording. A bipolar stimulating electrode was placed into Schaffer collateral pathway of the dorsal hippocampus (4.5 mm posteriorly to bregma, 3.7 mm laterally to the midline, and at 2.5 mm depth from the brain surface). A recording electrode was positioned in the ipsilateral stratum radiatum underneath the CA1 area (3.5 mm posteriorly to bregma, 2.5 mm laterally, and at 2.0 mm depth from the brain surface). A 30 min rest period was implemented after the electrodes insertion.

Baseline responses were collected by low-frequency stimulation (0.05 Hz, width 0.1 ms, and 50% of maximal response) and recorded for 20 min. Subsequently, four 100 Hz trains of high-frequency stimulation (HFS, 80% of maximum response) were delivered, including 20 pulses and 30 s of intertrain intervals, lasting for an additional 20 min. Following HFS, field excitatory postsynaptic potentials (fEPSPs) were recorded, being set at 5 Hz at the same stimulus intensity as the baseline pulse, over 60 min. fEPSP slope level was calculated by the ratio of the absolute fEPSP slope to baseline value. It was defined that LTP was successfully induced if the fEPSP slope level was ≥120%. Evoked field responses were acquired, amplified, monitored, and analyzed with RM6240BD biology signal processing system (Chengdu Instrument Factory, China).

2.6. Golgi Silver Stain. Under anesthesia (10% chloral hydrate, 350 mg/kg, ip), rats underwent transcardial perfusion with 0.9% saline solution (containing 0.5% sodium nitrite), followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) for 2 h. Then the rats were perfused with a mordant dye comprising a mixture of 5% chloral hydrate, 5% potassium dichromate, 4% formaldehyde, and distilled water for another 1-2 h in dark until toes of the perfused rats turned to be heavy tangerine. Brains were taken out and coronally sectioned to three pieces, of which the middle ones were soaked in the mordant agent for 3 days. Consequently, the specimens were immersed in 1.5% silver nitrate solution for another 3 days. Finally, the brain sections were cut into 50 μm slices on a vibratome (Campden Instrument, MA752, Leicester, UK) and rinsed with 2% potassium dichromate solution, dehydrated, cleared, and coverslipped.

Using Olympus BX51 microscope, images of dendrites of pyramidal neurons in the hippocampal CA1 region were traced for at least 50 μm from secondary and tertiary dendritic branches at 1000x magnification. The density of dendritic spines along the length was estimated by spine number/10 μm.

2.7. Histology. Under anesthesia, rats were perfused with 0.9% saline solution followed by 4% PFA in 0.1 M phosphate buffer, pH 7.4. Brains were then removed and fixed for 6–8 h at 4°C. Following paraffin embedding, from each rat were collected three coronal slices containing the dorsal hippocampus at 5 μm thickness. Toluidine blue staining (Nissl staining) was performed. Representative photomicrographs of pyramidal cell layer of CA1 region were microscopically captured. Quantitative analysis of the ratios of viable neurons was processed by Image-Pro Plus (Leica DMLB) software at 400x magnification. An average of three Nissl-stained sections was calculated to yield the single parameter per rat. All microscopic analyses were conducted by an observer blinded to the groups.

2.8. Western Blot. Rats in each group were sacrificed by decapitation. Hippocampus tissues were quickly removed from the brain and homogenized in ice-cold lysate buffer containing 50 mmol/L Tris-HCl (pH = 8.0), 150 mmol/L NaCl, 1% Triton X-100, 100 μg/mL PMSF, and phosphatase inhibitors (Sigma, USA). Proteins were fractionated on 10% SDS-PAGE gels and then transferred to polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Germany). The blots were then incubated with phospho-CREB (Ser133) rabbit anti-rat monoclonal antibody (mAb) (1 : 1000, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-rat polyclonal antibody for BDNF (1 : 300, Boster, Wuhan, China), goat anti-rat polyclonal antibody for p250GAP (1 : 200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), or β-tubulin mouse anti-rat mAb (1 : 1000, Affinity Bioscience, USA) antibodies overnight at 4°C. The immunocomplexes were visualized with horseradish peroxidase (HRP) conjugated mouse anti-rabbit, mouse anti-goat, or rabbit anti-mouse secondary antibodies (1 : 5000, Proteintech Group Inc., Wuhan, China) by using an enhanced chemiluminescence (ECL) system (Millipore, Billerica, MA, US). Optical density of the bands was scanned and quantified with NIH ImageJ software and the results were normalized to the quantity of β-tubulin in each sample lane. All assays were repeated at least three times.

2.9. Quantitative Real-Time PCR. Hippocampal tissues were processed with RNAlater® Solution (Ambion® RNA by Life Technologies™, New York, USA) to stabilize and protect RNA before storage in liquid Nitrogen. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). 10 ng RNA was reverse-transcribed into cDNA with the Toyobo First-Strand cDNA Synthesis Kit (USA). U6 RNA served as an endogenous reference. The reactions were carried out in triplicate in a StepOne™ Real-Time PCR System (Life Technologies, New York, USA) in a 10 μL reaction mixture. The relative change of miR132 expression was determined using $2^{-\Delta\Delta CT}$ method [24]. The primers for pre-miR132 were as follows: forward primer: 5′-ACCGTGGCTTTCGATTGTTAC-3′ and reverse primer: 5′-TGGTGTCGTGGAGTCG-3′, and those for U6 were as follows: forward primer:

5′-CCTGCTTCGGCAGCACA-3′ and reverse primer: 5′-AACGCTTCACGAATTTGCGT-3′.

2.10. Statistical Analysis. All data were presented as mean ± SEM and statistically analyzed with SPSS 19.0 software (IBM Corporation, Somers, New York, USA). The escape latencies in MWM test were tested by two-way ANOVA with repeated measures. The other data were analyzed by one-way ANOVA followed by post hoc test for multiple comparisons among the control group, model group, and EA group and were evaluated by independent-samples Student's t-test between the EA+H89 group and EA+NS group. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. EA Ameliorated Spatial Learning and Memory Deficits Induced by 2VO, and the Effect Was Partially Inhibited by H89. The potential protective effects of EA against spatial learning and memory deficits of 2VO rats were assessed using the MWM test. The analysis of escape latencies showed significant differences between the control group, model group, and EA group (group effects: $F(2, 65) = 19.489$, $P = 0.000$; training day effects: $F(4, 260) = 13.239$, $P = 0.000$; and $n = 10$ per group). Compared with the control group, the model group rats exhibited significantly longer escape latencies from the 2nd to the 5th time during the training period ($P < 0.05$, resp., Figure 2(b)). Compared with the model group, the EA group rats shortened the escape latencies from the 3rd to the 5th time during the training period ($P < 0.05$, resp., Figure 2(b)). The analysis of escape latencies showed significant differences between the EA+H89 group and EA+NS group (group effects: $F(1, 38) = 18.913$, $P = 0.000$; training day effects: $F(4, 152) = 12.944$, $P = 0.000$; and $n = 10$ per group). The EA+H89 group rats demonstrated significantly prolonged escape latencies than the EA+NS group rats from the 2nd to the 5th time during the training period ($P < 0.05$, resp., Figure 2(c)). Typical swimming paths in the spatial probe trial of each group were displayed in Figure 2(d). Analyses with one-way ANOVA revealed a significant difference between the control group, model group, and EA group ($F(2, 27) = 5.470$, $P = 0.010$; $n = 10$ per group). The time spent in the target quadrant was significantly decreased in the model group ($P < 0.01$ versus control group, Figure 2(e)), while it was significantly increased in the EA group ($P < 0.01$ versus model group, Figure 2(e)). The EA+H89 group rats exhibited significantly shorter time than that of the EA+NS group ($t(9) = -2.902$, $P < 0.01$ versus EA+NS group; $n = 10$ per group, Figure 2(f)).

3.2. EA Alleviated LTP Impairment and the Reduction of Dendritic Spine Density of 2VO Rats, and the Effect Was Partially Inhibited by H89. The analysis of fEPSP slopes showed a significant difference among the control group, model group, and EA group ($F(2, 90) = 112.541$, $P = 0.000$; $n = 10$ per group). As shown in Figures 3(a) and 3(b), HFS of the Schaffer collateral inputs to CA1 pyramidal cells induced a stable LTP in the slope of fEPSP in control rats (20–50 min after HFS: 252.22 ± 7.98% of baseline values; 50–80 min after HFS: 208.46 ± 5.91% of baseline values). Contrastingly, in the model group, fEPSP slope was significantly reduced (20–50 min after HFS: 144.88 ± 7.46% of baseline values; 50–80 min after HFS: 111.37 ± 4.66% of baseline values, $P < 0.01$ versus the control group). EA reversed the 2VO-induced LTP impairment (20–50 min after HFS: 204.19 ± 7.58% of baseline values; 50–80 min after HFS: 162.45 ± 6.37% of baseline values, $P < 0.01$ versus the model group). The normalized fEPSP slope was significantly decreased in EA+H89 group (20–50 min after HFS: 144.92 ± 6.50% of baseline values; 50–80 min after HFS: 123.52 ± 7.05% of baseline values; $t(60) = -10.534$, $P = 0.000$ versus EA+NS group; and $n = 10$ per group, Figures 3(c) and 3(d)) compared to that in the EA+NS group (20–50 min after HFS: 207.48 ± 6.42% of baseline values; 50–80 min after HFS: 171.56 ± 8.16% of baseline values).

The analysis of dendritic spine density showed a significant difference among the control group, model group, and EA group ($F(2, 54) = 58.820$, $P = 0.000$; $n = 6$ per group). The dendritic spine density was markedly decreased in model group rats (control rats: 11.29 ± 0.52, model rats: 6.21 ± 0.27, $P < 0.01$ versus the control group, Figure 3(f)), and it was significantly improved in EA group rats (EA rats: 9.21 ± 0.22, $P < 0.01$ versus the model group). In addition, the EA+H89 group rats had significantly fewer dendritic spines than those of the EA+NS group rats (5.20 ± 0.44 and 7.98 ± 0.67, resp.; $t(19) = -3.821$, $P < 0.01$ versus the EA+NS group, Figure 3(f)).

3.3. Significant Signs of Neuronal Loss Were Absent in Hippocampus in Experimental Rats from the Five Groups. Nissl stain was used to distinguish viable neurons from the apoptotic or the neurotic ones. The former exhibited abundant cytoplasm and Nissl substance (stained as dark blue), obvious oval nuclei (stained as light blue), and prominent nucleoli while the apoptotic or necrotic cells exhibited pyknotic morphology with amorphous or fragmented nuclei.

Significant signs of neuronal loss were absent according to the overall observation in the hippocampus of experimental rats from the five groups (Figures 4(a)–4(e)). Considering previous reports that CA1 region is more vulnerable to ischemic damage [25], we focused on the neuronal damage in CA1 pyramidal cell layer. As shown in Figures 4(a1)–4(e1), several damaged neurons (black arrows) in CA1 pyramidal cell layer existed in the sections from the five groups. In Figure 4(f), quantitative and statistical analysis of pyramidal cell viability in CA1 pyramidal cell layer did not reach significance neither among control rats, model rats, and EA rats (control: 65.19 ± 1.25, model: 59.70 ± 3.20, and EA: 61.34 ± 1.15; $F(2, 109) = 3.075$, $P > 0.05$; and $n = 5$ per group) nor between EA+H89 rats and EA+NS rats (EA+H89: 56.07 ± 1.77, EA+NS: 56.69 ± 0.93, $t(26) = -0.336$, $P > 0.05$; and $n = 5$ per group).

3.4. EA Increased the Expression of pCREB Protein, BDNF Protein, and miR132 and Decreased the Expression of p250GAP Protein in the Hippocampus of 2VO Rats and These Effects Were Partially Inhibited by H89. Analyses with one-way ANOVA revealed a significant main effect of EA (pCREB: $F(2, 17) = 11.766$, $P < 0.01$; BDNF: $F(2, 19) = 13.820$, $P = 0.000$; miR132: $F(2, 33) = 9.106$, $P < 0.01$; and p250GAP:

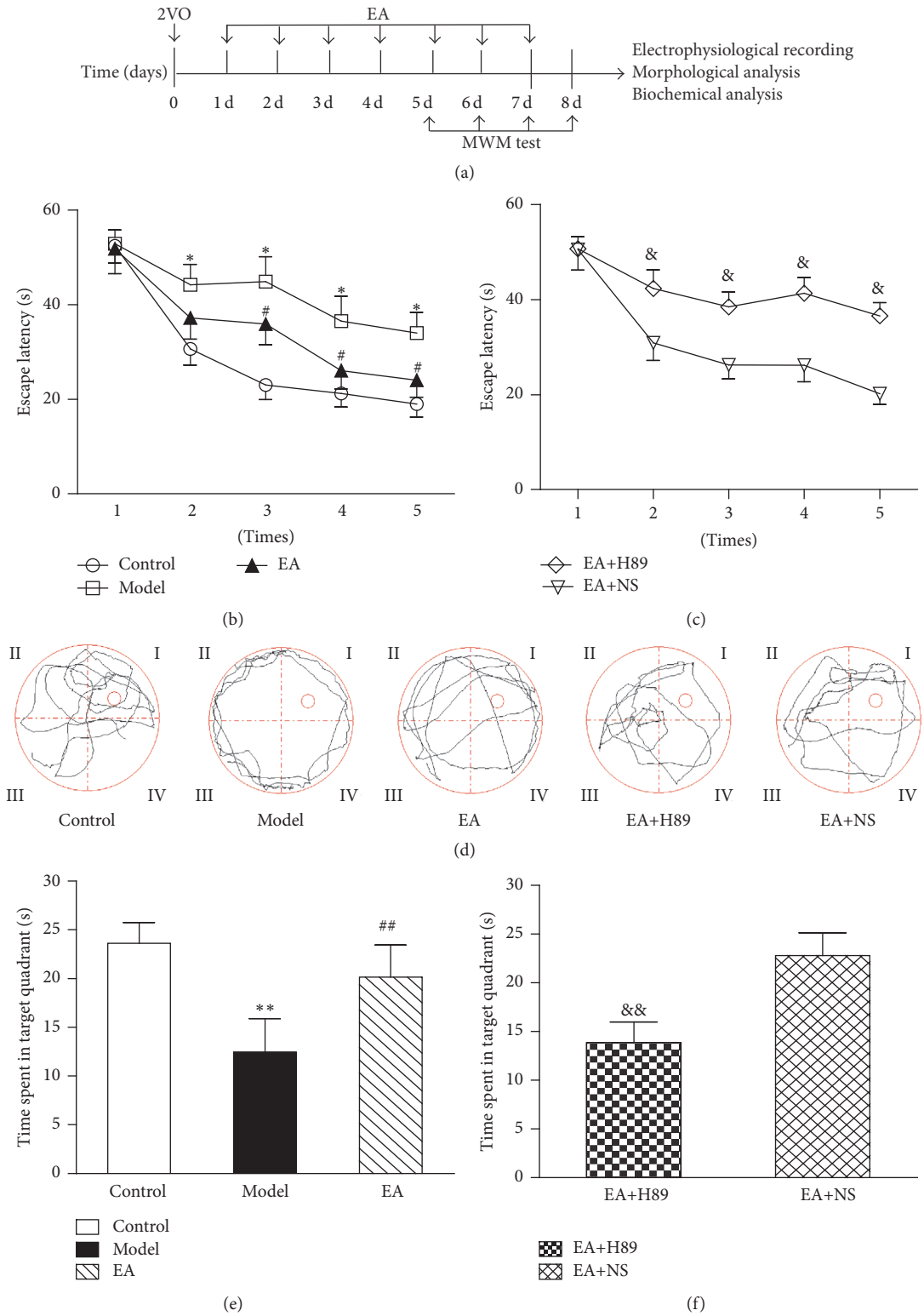

Figure 2: EA prevented spatial learning and memory deficits induced by 2VO, and the effect was partially inhibited by H89. (a) Timeline of the experiments to show the EA therapy and detection. (b and c) Escape latency to find the hidden platform from the 1st to the 5th time. Rats in the model group had longer escape latencies than the control group (b). EA could reverse the spatial learning impairment induced by 2VO. Rats in the EA+H89 group had longer escape latency than the EA+NS group rats (c). H89 could partly reverse the beneficial effect of EA. (d) The typical swimming paths of each group in the spatial probe trial. (e and f) Time spent in the target quadrant in the probe test. The amount of time expended in the target quadrant was significantly shorter in the model group rats than in the control group rats, and rats treated with EA exhibited significantly more time in the target quadrant (e). Rats in the EA+H89 group spent a shorter time in the target quadrant compared to EA+NS group rats (f). Each value represents mean ± SEM, $n = 10$ for each group. $^{**}P < 0.01$, compared with the control group; $^{*}P < 0.05$, compared with the control group; $^{\#\#}P < 0.01$, compared with the model group; $^{\#}P < 0.05$, compared with the model group; $^{\&\&}P < 0.01$, compared with the EA+NS group; and $^{\&}P < 0.05$, compared with the EA+NS group.

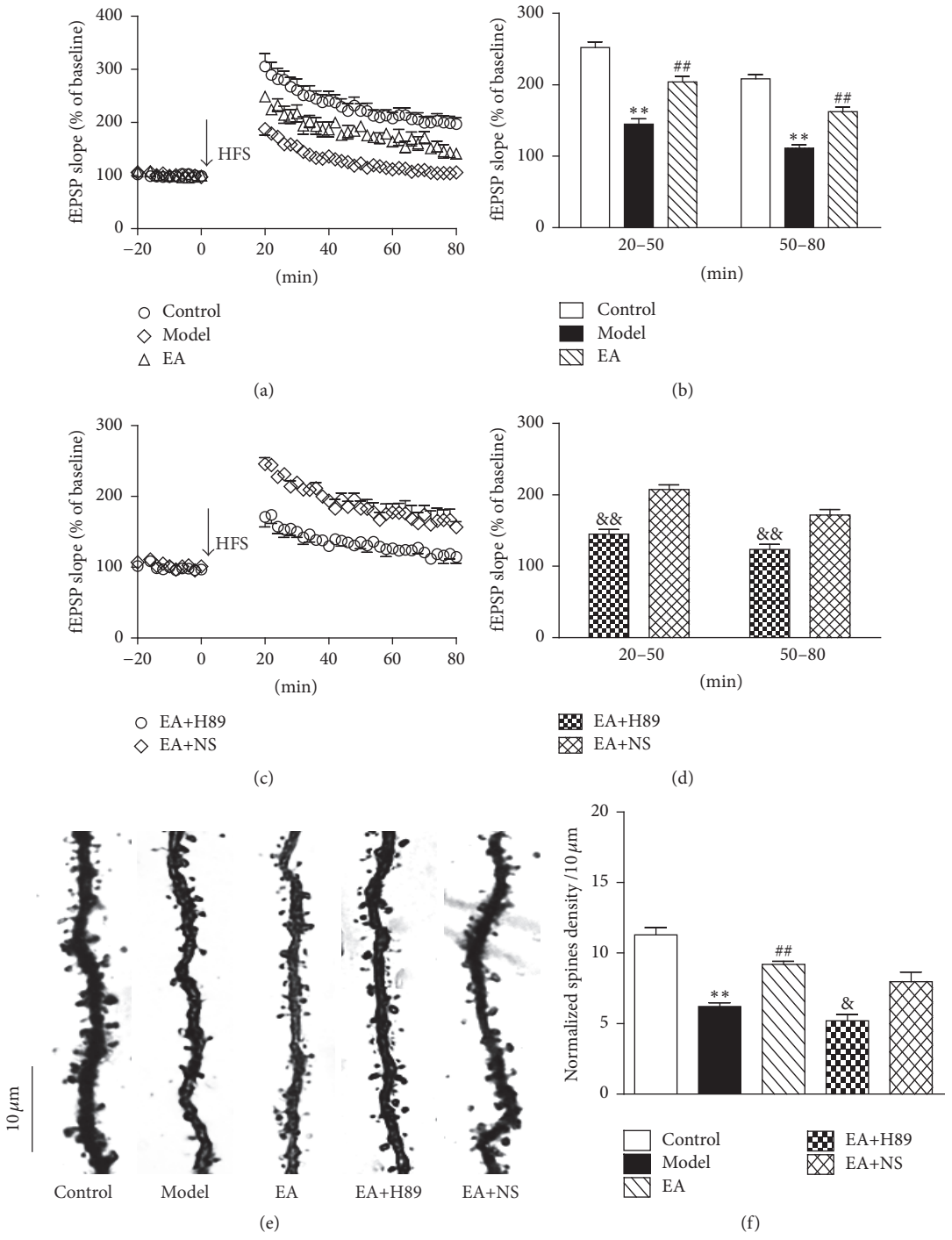

Figure 3: EA alleviated LTP impairment and the reduction of dendritic spine density of 2VO rats, and the effects were partially inhibited by H89. (a and c) The linear graph of the normalized fEPSP slope. The downward filled arrow indicates HFS. (b and d) The histogram of average fEPSP slope at 20–50 min and 50–80 min after HFS. The normalized fEPSP slope was significantly decreased in the model group. EA reversed the 2VO-induced LTP impairment, and this effect was partly inhibited by H89. Each value represents mean ± SEM, $n = 10$ for each group. (e) Representative dendritic segments of CA1 pyramidal neurons in the hippocampus (1000x, scale bar = 10 μm). (f) Normalized mean dendrite spine density counts. EA attenuates 2VO-induced dendritic spine loss of CA1 region in the hippocampus, and this effect was partly inhibited by H89. Each value represents mean ± SEM, $n = 6$ for each group. $^{**}P < 0.01$, compared with the control group; $^{\#\#}P < 0.01$, compared with the model group; $^{\&\&}P < 0.01$, compared with the EA+NS group; and $^{\&}P < 0.05$, compared with the EA+NS group.

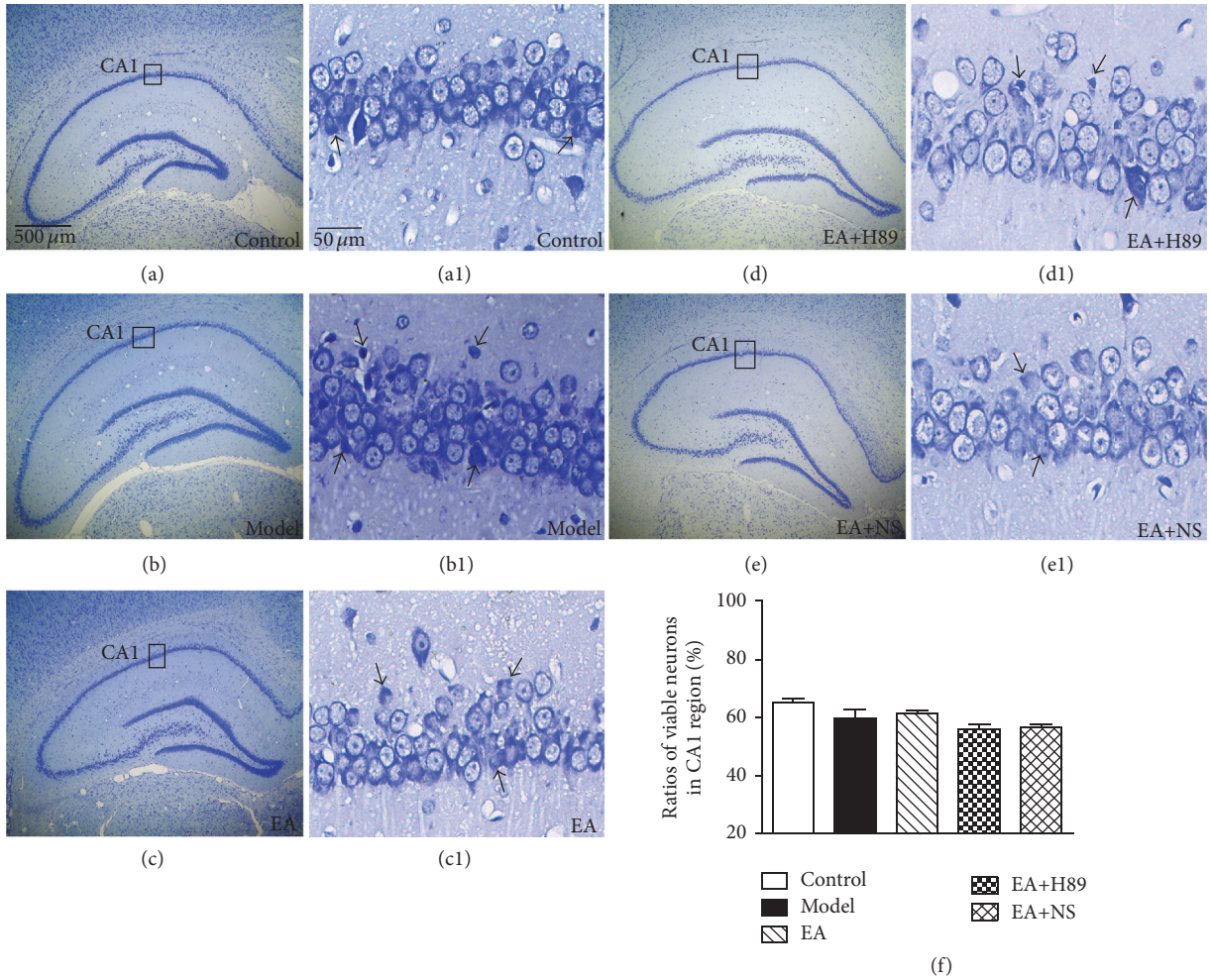

FIGURE 4: Significant signs of neuronal loss were absent in hippocampus in experimental rats from the five groups. Photomicrographs of toluidine blue-stained sections from control group (a and a1), model group (b and b1), EA group (c and c1), EA+H89 group (d and d1), and EA+NS group (e and e1). (a–e) 40x, scale bar = 500 μm. (a1–e1) 400x, scale bar = 50 μm. Significant signs of neuronal loss were absent based on the overall observation of hippocampus in experimental rats from the five groups. Several damaged neurons (black arrows) in CA1 pyramidal cell layer were observed. (f) Quantitative analysis of pyramidal cell viability in CA1 pyramidal cell layer was not significantly different neither among the control rats, the model rats, and the EA rats (control: 65.19 ± 1.25, model: 59.70 ± 3.20, and EA: 61.34 ± 1.15; $F(2, 109) = 3.075$, $P > 0.05$; and $n = 5$ per group) nor between the EA+H89 rats and the EA+NS rats (EA+H89: 56.07 ± 1.77, EA+NS: 56.69 ± 0.93; $t(26) = -0.336$, $P > 0.05$; and $n = 5$ per group). Each value represents mean ± SEM.

$F(2, 17) = 10.539$, $P < 0.01$; and $n = 5$ per group). As illustrated in Figure 5, the expressions of the pCREB protein, BDNF protein, and miR132 were decreased 7 days after the 2VO operation, accompanied by an increased expression of p250GAP protein (pCREB, BDNF, miR132, and p250GAP: $P < 0.01$, resp., versus the control group). Treatment of EA impeded the reduction of pCREB protein, BDNF protein, and miR132 expression in 2VO rats, as well as suppressing the increase of p250GAP protein (pCREB, BDNF, miR132, and p250GAP: $P < 0.05$, resp., versus the model group). Moreover, this protective effect of EA was partially reversed in EA+H89 group rats, when compared with the rats in EA+NS group (pCREB: $t(4) = -3.290$, $P < 0.05$; BDNF: $t(7) = -2.711$, $P < 0.05$; miR132: $t(17) = -2.109$, $P < 0.05$; and p250GAP: $t(7) = 2.722$, $P < 0.05$, versus the EA+NS group; and $n = 5$ per group, Figure 5).

4. Discussion

The present study has revealed that EA at GV20 and GV14 for 7 days could significantly ameliorate learning and memory deficits and LTP impairment in the Schaffer collateral pathway. In addition, it could also restore the dendritic spine loss in the hippocampal CA1 region in cerebral hypoperfusion rats. PKA/CREB signaling pathway was potentially involved in the neuroprotective effects, including the regulation of proteins such as pCREB, BDNF, p250GAP, and miR132.

The 2VO rat model is widely used to study the chronic cerebral hypoperfusion- (CCH-) related cognitive impairment. The subchronic phase of this model was defined as 3 days–8 weeks after the occlusion, causing a dramatic reduction in cerebral blood flow (CBF) and hypoxic-ischemic conditions [26]. Even if the CBF gradually returns to the baseline

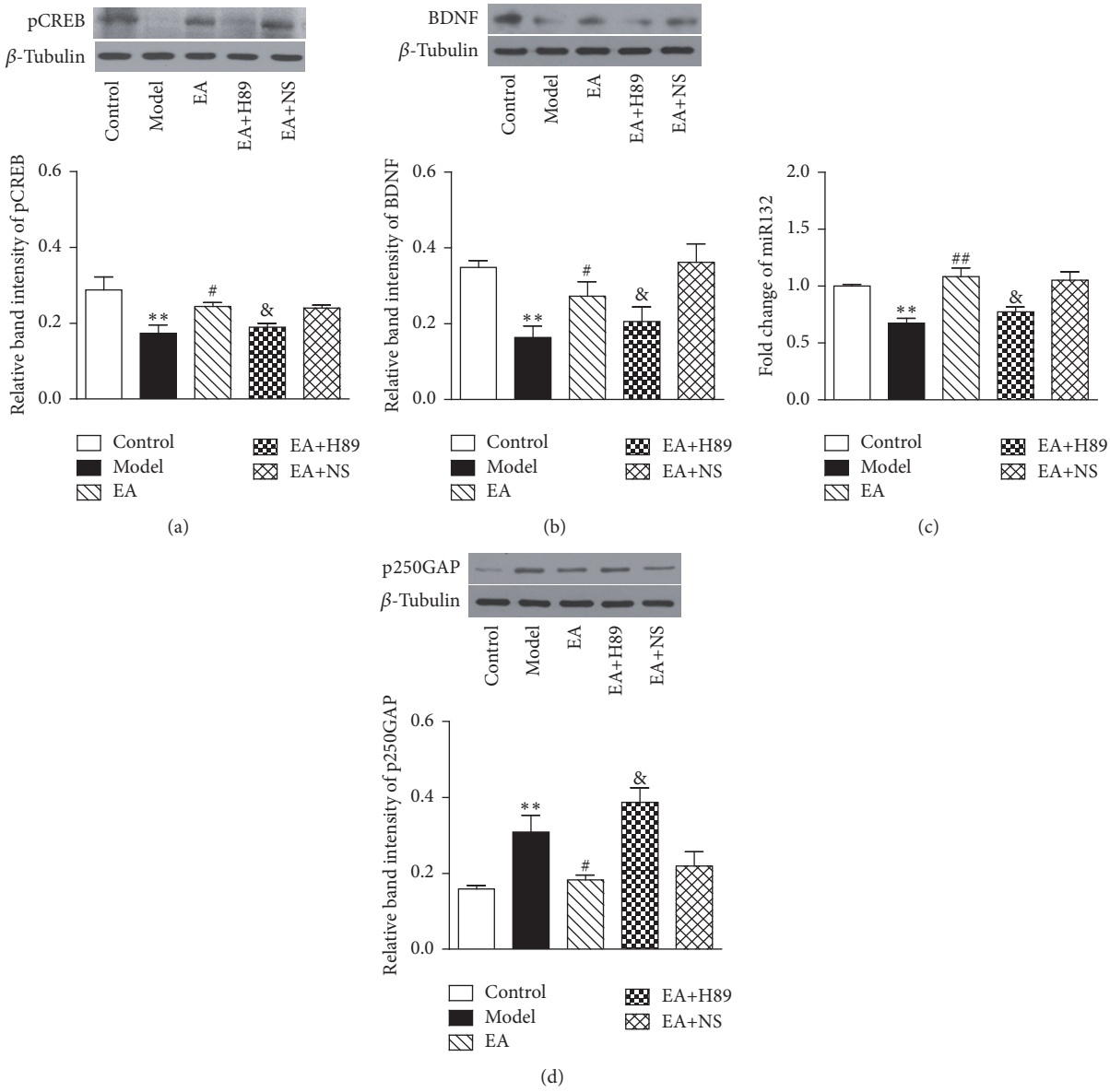

Figure 5: EA increased the expression of the pCREB protein, BDNF protein, and miR132 and decreased the expression of p250GAP protein in the hippocampus of 2VO rats and these effects were partially inhibited by H89. (a) Immunoblot analysis for pCREB protein in hippocampus and histogram of its relative band intensity. (b) Immunoblot analysis of BDNF protein in hippocampus and histogram of its relative band intensity. (c) Histogram of fold change of miR132 in the hippocampus. (d) Immunoblot analysis for p250GAP protein in hippocampus and histogram of its relative band intensity. Quantitative analysis demonstrated that hippocampal pCREB, BDNF, and miR132 levels were decreased by 2VO and increased by EA treatment. Concurrently, EA reduced the elevation of p250GAP level in the hippocampus of 2VO rats. These protective effects were at least partially inhibited by H89 administration. Each value represents mean ± SEM, $n = 5$ for each group. $^{**}P < 0.01$, compared with the control group; $^{\#\#}P < 0.01$, compared with the model group; $^{\#}P < 0.05$, compared with the model group; and $^{\&}P < 0.05$, compared with the EA+NS group.

with the compensatory mechanism of vascular plasticity, such cerebral hypoperfusion may be a risk factor for subsequent neuronal degeneration and cognitive impairment [27]. In Schaffer-CA1 synapses, the LTP was significantly inhibited at both 1 day and 4 days after clamping of the bilateral common carotid arteries; that is, a so-called delayed dysfunction might exist in the hippocampal neurons [28]. Consecutively, it was demonstrated that the synaptic transmission reduction of the hippocampus and prefrontal cortex was partially attributed to the dendritic spines loss in the hippocampus, which contributed to the learning and memory dysfunctions in 2VO rats [29]. Therefore, interventions that reverse, at least partly, early neuropathological outcomes of CCH might be an effective way to prevent the progressive decline of cognitive functions. In this study, EA could significantly reverse the LTP impairment and dendritic spine loss, indicating a beneficial role in improving the neuronal synaptic transmission and functional reconstruction in cerebral hypoperfusion rats.

Significant pathological changes in the brain may be absent at early stages of bilateral common carotid ligation. A previous study had shown that the pyramidal cell loss in the unilateral hippocampal CA1 region was only observed in a few 2VO rats (1/6) on day 7 after the surgical procedure [30]. Similarly, it was reported that 2VO did not influence the hippocampal CA1 pyramidal cell number or density of glial fibrillary acidic protein (GFAP) 14 days after surgery, though a late-emerging CA1 cell loss was detected at 190 days after the operation [31]. Together, these findings have prompted a consensus that initial 2VO does not cause significant neuronal loss. Consistent with these reports, our experimental outcomes also detected minimal differences in viable hippocampal neurons among the experimental rats.

CREB is best known for its roles in learning and memory. Over the past few decades, accumulated evidence established that pCREB-mediated gene transcription and protein synthesis contributed to the development of long-term memory [32]. This was brought about by participating in processes such as long-term potentiation of synaptic strength [15, 32] and structural synaptic changes. In a gerbil global ischemia model, preconditioning ischemia induced ischemic tolerance by a transient increase of pCREB and subsequent upregulation of BCL-2 expression [33]. However, ongoing chronic ischemia reduced the expression of pCREB, resulting in the selective vulnerability of CA1 pyramidal cells at 48 and 72 h following mild hypoxic-ischemic (HI) injury in rats [25]. In the present study, we found that EA could increase the expression of the pCREB protein in the hippocampus of 2VO rats, suggesting that EA might improve learning and memory through activation of CREB mediating gene transcription and protein synthesis.

Sufficient evidences have suggested that CREB is a major regulator of BDNF-induced neuronal responses. On one hand, the transcription and translation of BDNF gene are CREB-dependent [34, 35]; on the other hand, BDNF could stimulate Ser133 phosphorylation and activate CREB through CaMKIV [36] and RSKs [37]. Thus, a positive feedback loop might have connected BDNF and CREB. Meanwhile, BDNF was demonstrated to play a crucial role in synaptogenesis [38] and LTP [39, 40]. Moreover, it was reported to enhance high-frequency transmission [41] and mediate the redistribution of the synaptic proteins within the presynaptic terminals. Similar findings were observed in this study: that hippocampal BDNF protein expression was decreased after the 2VO procedure and increased after the EA treatment, accompanied by alleviation of LTP impairment and partial restoration of dendritic spine density.

We also observed another newly recognized CREB-driven target, miR132, which was indicated in mediating dendritic plasticity through suppressing translation of p250GAP, a member of the Rac/Rho family of GAPs [21, 42]. As reported, the transcription of miR132 was induced in response to neurotrophins [43] or synaptic activity [44]. BDNF could regulate miR132 transcription via the ERK1/2 signaling, together with kinase MSK1 and the phosphorylation of CREB [43]. Herein, we found that EA altered the morphology of dendritic spines, accompanied by an increase of miRNA-132 and a reduction of p250GAP protein in 2VO rats. These results revealed the important role of miR132 in the improvement of learning and memory by EA.

A group of rats were pregiven H89 [45] through icv injection and then received operation of 2VO and EA treatments. Interesting results were found that H89 administration could reverse the beneficial effects of EA on spatial learning and memory, including LTP facilitation and restoration of dendritic spines. Simultaneously, the effects of EA on the molecular level of pCREB protein, BDNF protein, miR132, and p250GAP protein were also blocked by H89 in EA-treated 2VO rats. All these results implied a potential involvement of PKA/CREB signal pathway in the effects of EA on cognitive function and hippocampal synaptic plasticity in cerebral hypoperfusion.

5. Conclusions

Taken together, our data indicated that EA could ameliorate learning and memory deficits and alleviate hippocampal synaptic plasticity impairment of cerebral hypoperfusion rats, with a probable regulation of PKA/CREB signaling pathway, including the expression of pCREB protein, BDNF protein, miR132, and p250GAP protein.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Yi-Zhi-Fang-Dai Formula Protects against Aβ_{1-42} Oligomer Induced Cell Damage via Increasing Hsp70 and Grp78 Expression in SH-SY5Y Cells

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Yi-Zhi-Fang-Dai formula (YZFDF) is an experiential prescription used to cure dementia cases like Alzheimer's disease (AD). In this study, the main effective compounds of YZFDF have been identified from this formula, and the neuroprotective effect against Aβ_{1-42} oligomer of YZFDF has been tested in SH-SY5Y cells. Our results showed that YZFDF could increase cell viability and could attenuate endothelial reticula- (ER-) mediated apoptosis. Evidence indicated that protein folding and endothelial reticula stress (ERS) played an important role in the AD pathological mechanism. We further explored the expression of Hsp70, an important molecular chaperon facilitating the folding of other proteins, and Grp78, the marker protein of ERS in SH-SY5Y cells. Data told us that YZFDF pretreatment could influence the mRNA and protein expression of these two proteins. At last, we also found that YZFDF pretreatment could activate Akt in SH-SY5Y cells. All these above indicate that YZFDF could be a potent therapeutic candidate for AD treatment.

1. Introduction

Alzheimer's disease (AD) is an age-related neurodegeneration disease which destroys cognitive function and eventually leads to death. Murray and colleagues [1] reported that AD had increased more in rank (from 32 to 9) of years lost to life because of premature mortality compared with any other major disease from 1990 to 2010. In the same year, a large-scale systematic analysis of the epidemiology of AD in China [2] showed that the incidence of dementia was 9.87 cases per 1000 person-years, and that of AD was 6.25 cases per 1000 person-years from 1990 to 2010. As there exists little treatment which can cure or slow down the progression of the disease, the socioeconomic impact of AD is growing steadily as the population is aging. The neuropathological features of AD are the formation of extracellular amyloid plaques (AP) composed of β-amyloid (Aβ) peptides [3, 4], the intracellular neurofibrillary tangles built by hyperphosphorylated Tau proteins [5, 6], and the loss of neurons [7]. The prevailing "amyloid cascade hypothesis" indicates that Aβ aggregation plays a critical role in the pathogenesis of AD [8–10]. Though the mechanisms underlying Aβ-mediated neurotoxicity still remain elusive, heat shock proteins are recognized as major contributors [11, 12].

Heat shock proteins (Hsps) are a class of molecular chaperons facilitating the folding of other proteins to ensure their maintenance of native conformation under stress or other toxic conditions [12, 13]. Heat shock protein 70 (Hsp70) is a major member of Hsps family and plays an important role in a complex neuroprotective system [11, 14]. Virally mediated Hsp70 overexpression rescued neurons from the toxic effects of intracellular Aβ accumulation [15], and exogenous Hsp70 can reduce Aβ plaque formation in 5XFAD mice [16]. Yurinskaya and his coworkers demonstrated that the effect of Hsp70 is realized via reduction of the oxidative stress and apoptosis induced by the peptide isoAsp7-Aβ(1–42) in human neuroblastoma cells [17]. Glucose-regulated protein 78 (Grp78) is the solo endoplasmic reticulum (ER)

homologue of Hsp70 and maintains the homeostasis of ER via participating in the process of protein folding and assembly and translocation of protein across the ER membrane. ER is an organelle coordinating synthesis, folding, exporting, and degradation of proteins, and evidence shows that endoplasmic reticulum stress (ERS) is closely related to AD [18, 19]. When misfolded proteins, like Aβ, accumulate in ER, Grp78 releases and activates ERS to restore the homeostasis of ER. However, when stress is prolonged or severe, cell apoptosis happens. Hsp70 can not only act as molecular chaperon helping proteins refold, but also enhance cells' ability to resist damage caused by oxidative stress [11, 15]. Moreover, evidence shows that Aβ can induce ERS and activate ER-related and mitochondria-related apoptosis [20, 21], so the problem here is to elucidate the role that Hsp70 and Grp78 play in Aβ-induced ERS and apoptosis.

Herbs have been widely used for thousands of years in China and with little serious side effects. Furthermore, compared with single-component drugs, Traditional Chinese Medicine (TCM) drugs exhibit a multicomponent, multipathway, and multitargets advantage and are able to treat multifactor, complex chronic diseases such as AD. Yi-Zhi-Fang-Dai formula (YZFDF), which is prescribed on the basis of clinical experience, is commonly used in clinic of TCM to treat dementia. YZFDF is composed of several compounds, including bilobalide, ginkgolide A, ginsenoside Rb1, ginsenoside Rg1, cistanoside A, and α-asarone. Our previous studies show that the extract of *Ginkgo biloba* leaves (EGb761), the main herb of YZFD formula, can protect against Aβ-induced cell damage [22, 23]. In a previous study, it has been reported that ginsenosides can restore metabolite imbalance in AD mice [24]. Wu et al. [25] suggested that *Cistanche tubulosa* extract could ameliorate the cognitive dysfunction in AD-like rat model. Moreover, α-asarone can inhibit proinflammatory cytokines and microglial activation in the hippocampus and ameliorate memory deficits [26].

This study aimed to examine the potential neuronal protective effect of YZFD formula against Aβ-induced neurotoxicity in SH-SY5Y cells. Our previous study indicates that Aβ_{1-42} oligomer showed more efficient neurotoxicity in SH-SY5Y cells than a scrambled Aβ_{42-1} peptide and 10 μM Aβ_{1-42} had a significant neurotoxicity effect on SH-SY5Y cells [22]; thus, we used 10 μM Aβ_{1-42} oligomer to treat cells mimicking AD cell damage model in this study. We examined the effects of YZFD formula on reducing Aβ-induced neurotoxicity through increasing the expression of Hsp70 and Grp78. In this study, our data support the possibility that YZFD formula might have protective effects, including the attenuation of neuron cell apoptosis and associated molecular chaperones.

2. Materials and Methods

2.1. Regents and Antibodies. Lyophilized human Aβ_{1-42} purified by HPLC was purchased from GL Biochem (Shanghai, China). We bought the rabbit anti-Hsp70, anti-Grp78, anti-caspase-12, anti-caspase-3, anti-p-Akt, and mouse anti-β-actin from Cell Signaling Technology (MA, USA) and rabbit anti-Akt1 from Millipore (MA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (CA, USA), and annexin V-FITC/PI Apoptosis Detection Kit was purchased from Beyotime (Shanghai, China).

2.2. Preparation of YZFDF Drug Powder. Four herbs were used in this study, including *Ginkgo biloba* leaves, ginseng, Cistanches Herba, and grass leaved sweetflag. All these herbs were purchased from Shanghai Hongqiao Pharmaceutical Co., Ltd. (Shanghai, China) and identified by TCM Preparation Room of Shanghai Geriatric Institute of Chinese Medicine, Shanghai University of Traditional Chinese Medicine. The main effective compounds of YZFDF were identified by Shanghai Jiao Tong University School of Pharmacy. The extracts of YZFDF were obtained as follows: 500 g of four herbs was subjected twice to extraction with 75% ethanol for 2 h. The dregs of the decoction were removed after filtering. The filtered liquid was concentrated by Rotary Evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland) and then dried using freeze drying method to get drug powder of 158.6 g. The YZFDF drug powder were stored at 4°C and dissolved in DMSO at a concentration of 200 mg/mL and then the required concentrations of YZFDF were prepared from the 200 mg/mL solution diluted in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA), a cell culture medium.

2.3. Identification of the Extracts of YZFDF. An Agilent 1100 HPLC system (Santa Clara, CA, USA) coupled with Fourier Transform Ion Cyclotron Resonance solariX 7.0T (Bruker Daltonics Inc., USA) and High Liquid Chromatography & Linear Ion Trap Quadrupole LTQ XL (Thermo Scientific, San Jose, CA, USA) were used for analysis of the extracts of YZFDF. Samples were prepared as follows: 3 g YZFDF drug powder was dissolved in 100 mL 70% ethanol with ultrasonic and then the extracted solution was dried with Rotary Evaporator. Stock solutions of main components of YZFDF were prepared in methanol and stored at 4°C. Working solutions were prepared on the day of analysis by further dilution of the stock solutions with methanol. The parameters for HPLC were as follows: for HPLC system coupled with solariX 7.0 T, chromatographic separation was performed on an Agilent ODS C18 column (50 mm × 2.0 mm, 5 μm) at room temperature and using a mobile phase that consisted of methanol-1% acetic aqueous solution (74 : 26) at a flow rate of 0.3 mL/min; for HPLC system coupled with LTQ XL, chromatographic separation was performed on an Agilent ODS C18 column (250 mm × 4.6 mm, 5 μm) at 35°C and the mobile phase was methanol in distilled deionized water (60/40, v/v) at a flow rate of 1.0 mL/min. The MS was operated with electrospray ionization (ESI) interface in positive and negative ionization mode for YZFDF. The ionization source conditions were capillary voltage 3.0 kV, cone voltage 55 V, source temperature 100°C, and desolvation temperature 250°C. The data was collected between 50 and 1000 m/z with the optimized collision energy at 6.0 V for YZFDF. The cone and desolvation gas flow rates were 50 and 600 L/h, respectively. The HPLC analysis spectrum is shown in Figure 1, while the chemical structures of each component and MS analysis spectrum are shown in Figure 2.

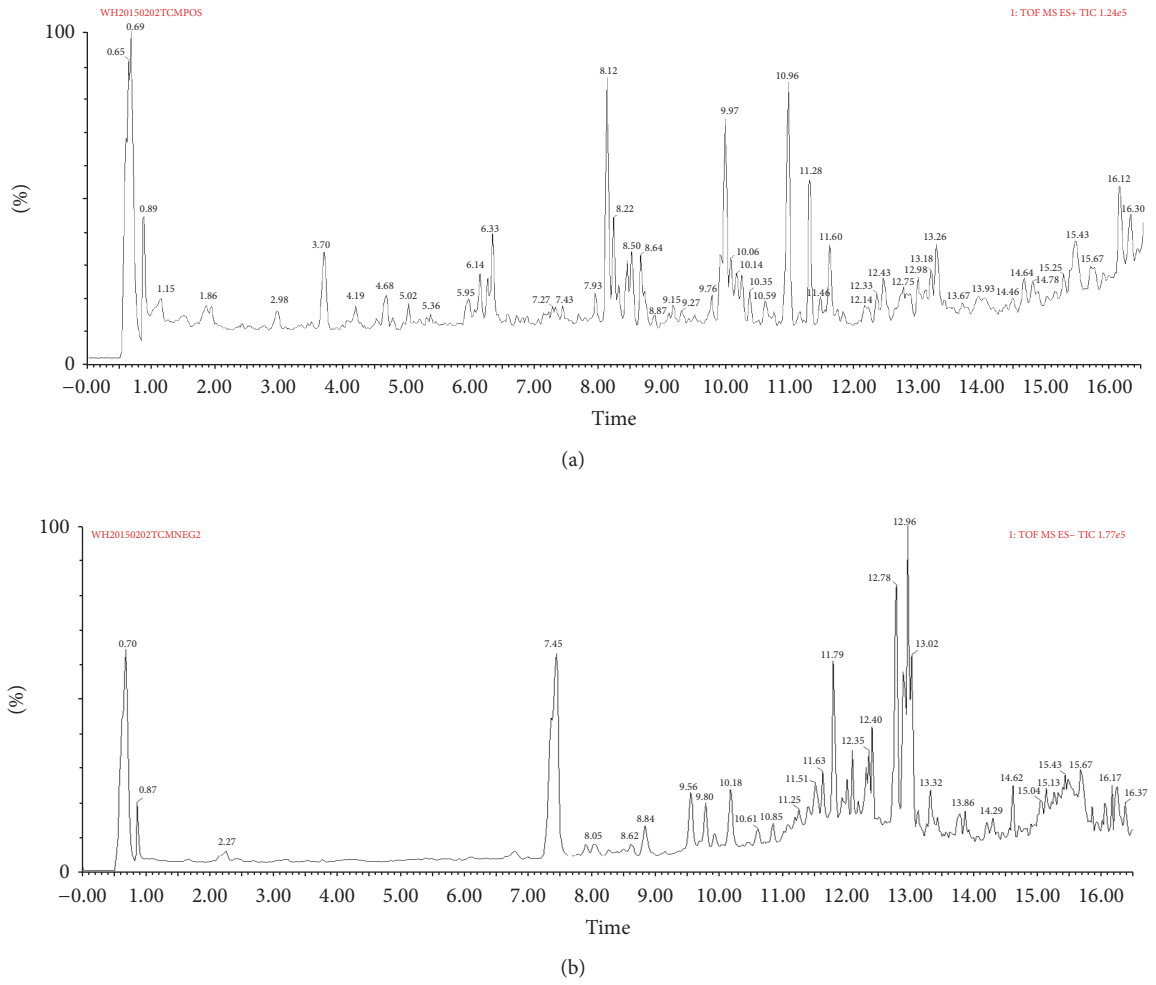


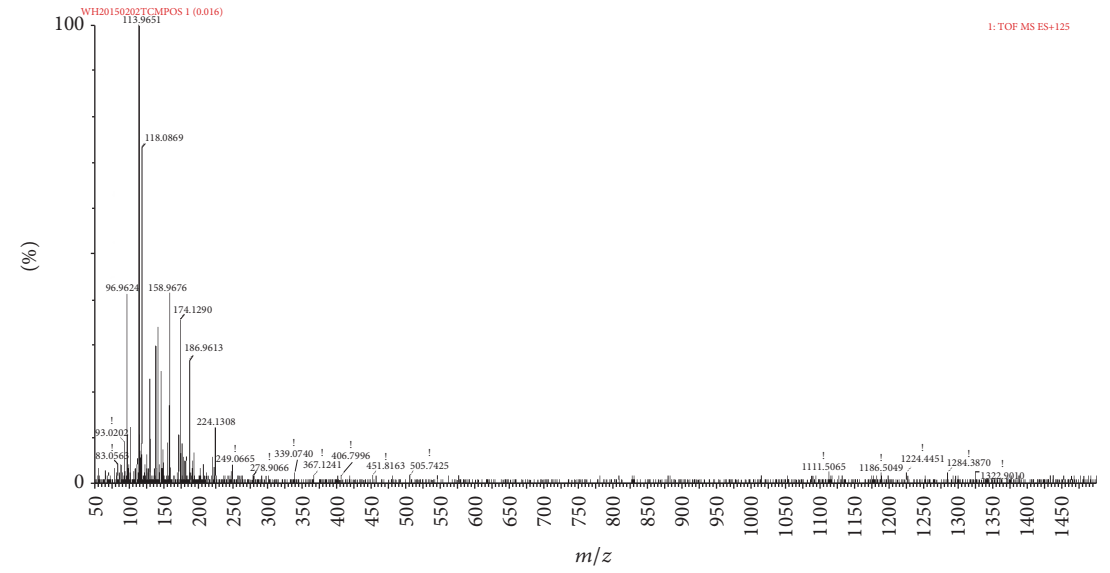
Figure 1: High-performance liquid chromatography (HPLC) analysis of Yi-Zhi-Fang-Dai formula (YZFDF). (a) The HPLC analysis spectrum of YZFDF in positive ion mode. (b) The HPLC analysis spectrum of YZFDF in negative ion mode.

2.4. Cell Culture and Treatments. Human neuroblastoma SH-SY5Y cells were grown and maintained in DMEM supplemented with glucose (4.5 g/L), fetal bovine serum (10%, Gibco, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C with 5% CO_2. The cells were pretreated with various concentrations of YZFDF for 2 h and then treated with 10 μM Aβ_{1-42} oligomer for 24 h. The 10 μM Aβ_{1-42} oligomers were prepared as mentioned previously [22]. In brief, 1 mg Aβ_{1-42} was initially diluted in 220 μL ice-cold hexafluoroisopropanol (HFIP, Sigma, USA) and then HFIP was removed under vacuum in a Speed Vac, and the peptide was stored at −20°C. For oligomer preparation, 2 mM Aβ_{1-42} dissolved in DMSO was diluted in ice-cold Opti-MEM (Gibco, USA) to bring the peptide to a final concentration of 100 μM and then incubated at 4°C for 24 h before use.

2.5. Cell Viability Assays. After various treatments, the viability of cells was determined by the MTT assay. In brief, cells were seeded on 96-well culture plates overnight, and then 20 μL MTT (5 mg/mL) was added to each well for 4 h at 37°C. 100 μL DMSO was added to solubilize the colored formazan product after the medium was aspirated. At last, the OD value of each well was detected at 490 nm using a microplate reader (BioTek, VT, USA). Cell viability (%) was expressed as a percentage relative to the untreated control cells.

2.6. Western Blotting. Following treatment, cells in each 6 cm culture plate were collected and the protein concentration was measured using the Bradford method. Equal amounts of protein were denatured and separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to nitrocellulose membranes (Millipore, USA) and were blocked with blocking buffer (Beyotime) for 1 h at room temperature. The membrane was incubated with primary antibodies (1 μg/mL) overnight at 4°C, followed by incubation with secondary antibody (1 μg/mL) (LI-COR, USA) at room temperature. Images were captured by the Odyssey infrared fluorescence imaging system (LI-COR, USA).

2.7. Quantitative Real-Time PCR (qRT-PCR). qRT-PCR assays were performed with the real-time PCR detection system (Eppendorf) using total RNA and the ReverTra Ace



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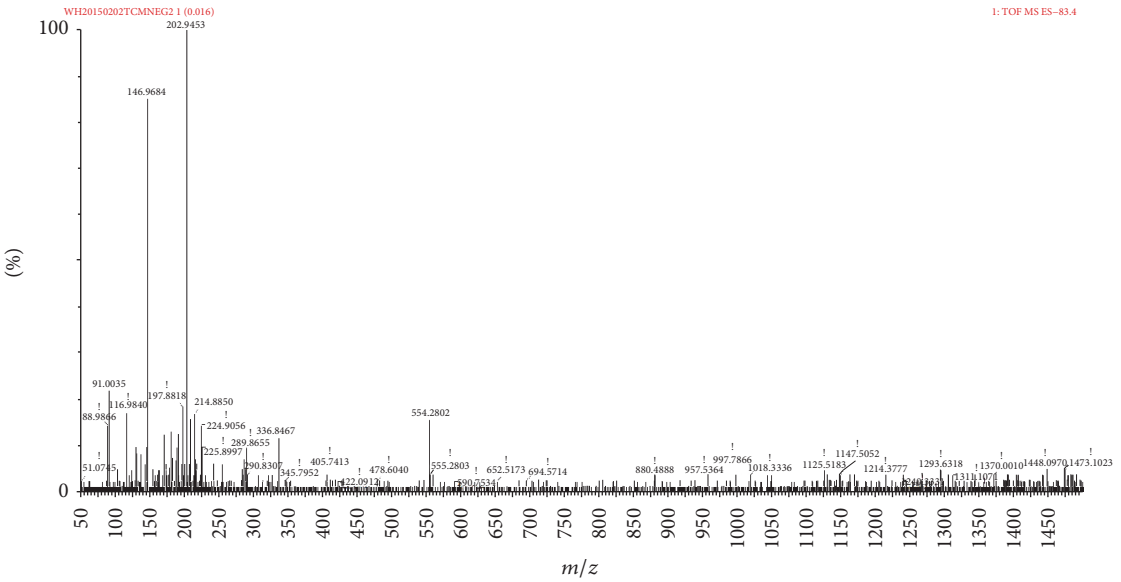

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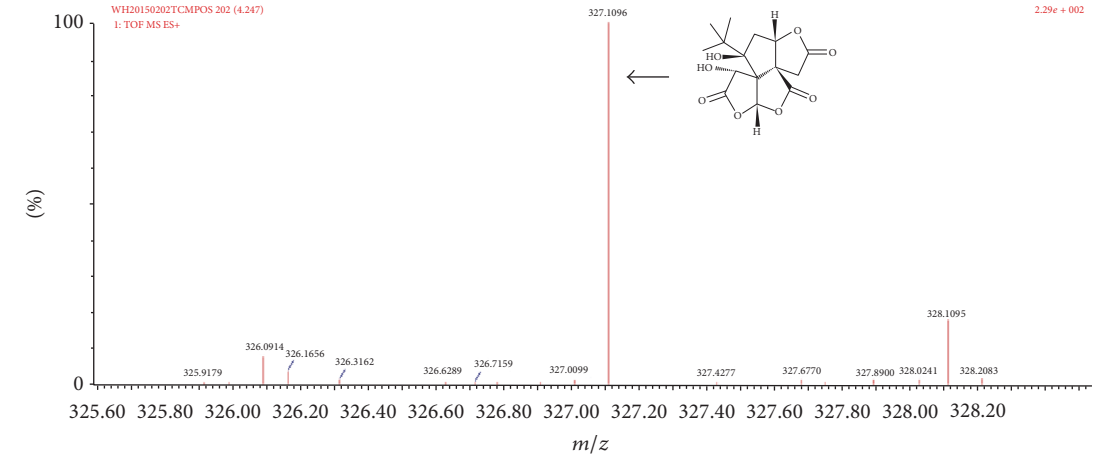

(c)

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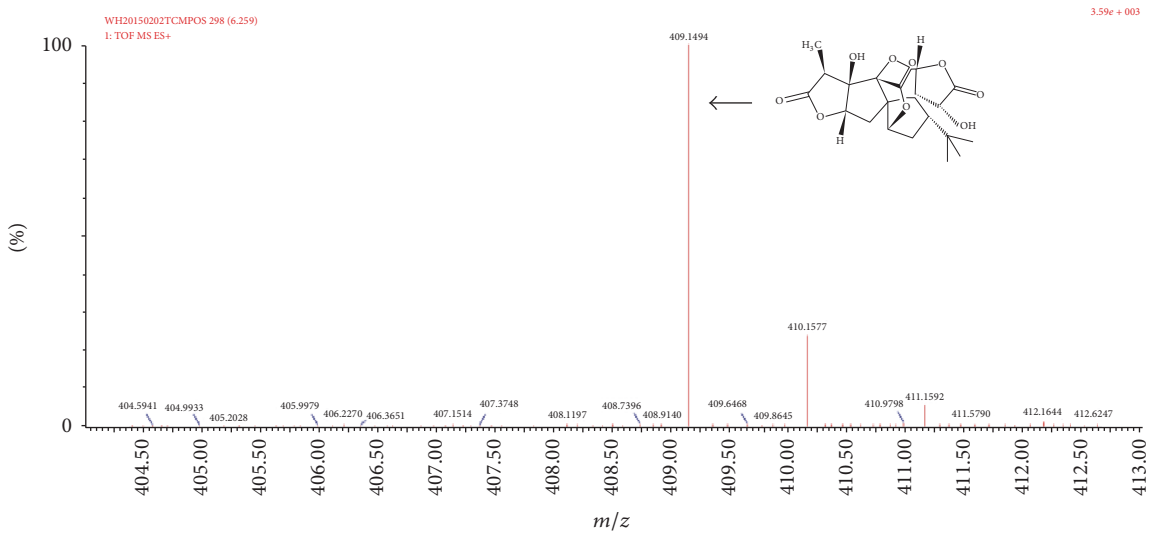

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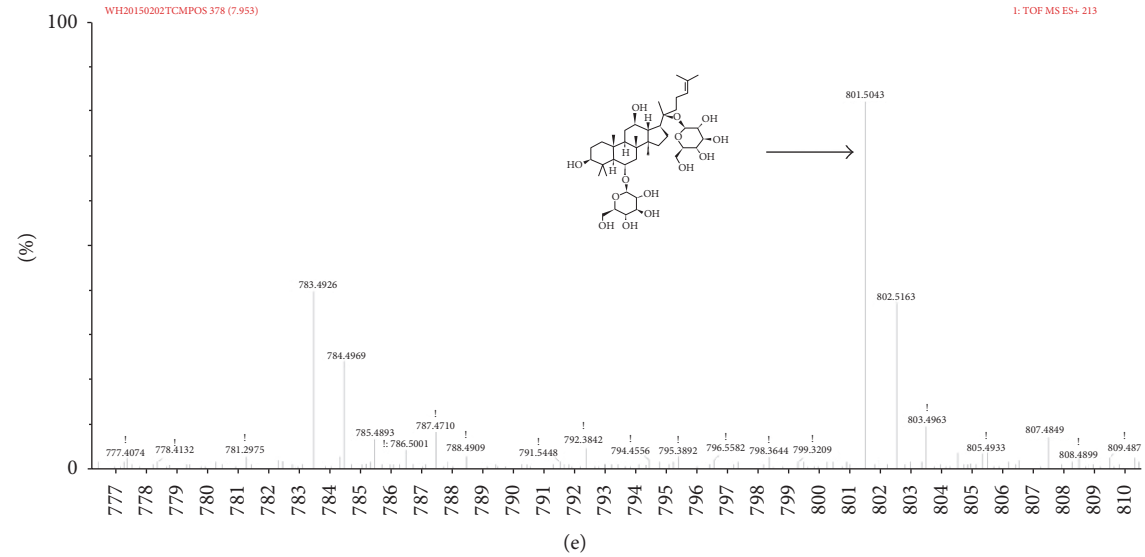

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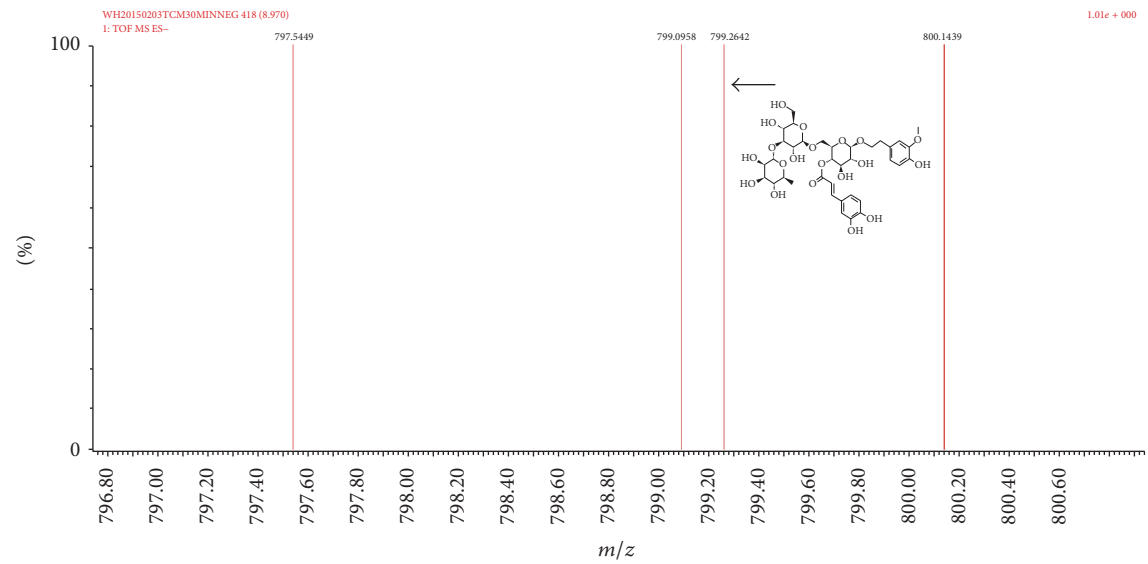

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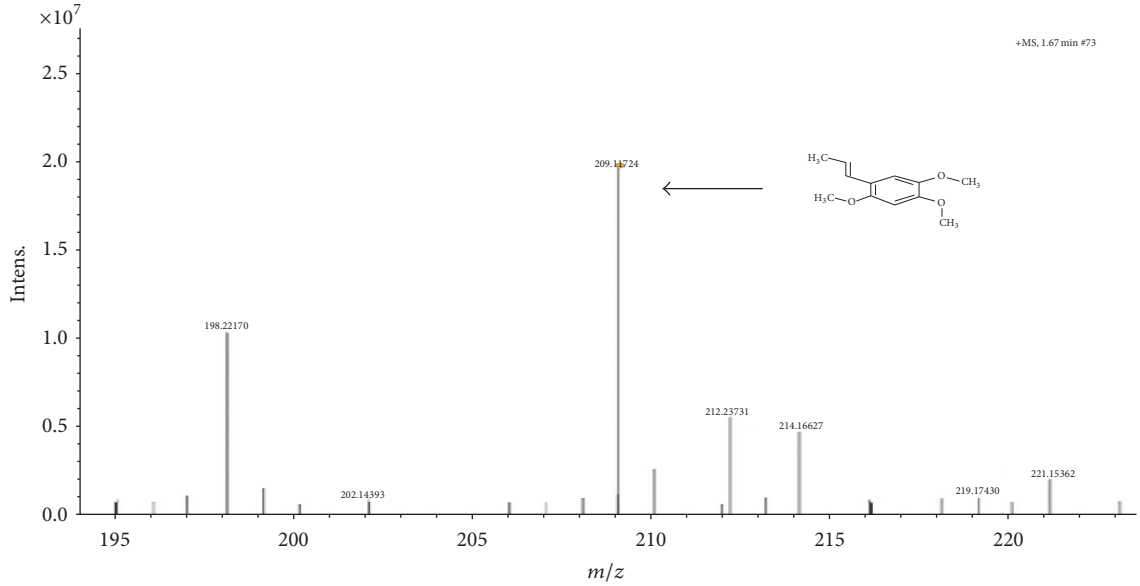


Figure 2: Chemical structures and mass spectrometry (MS) analysis spectrum of YZFDF. (a) The MS analysis spectrum of YZFDF in positive ion mode. (b) The MS analysis spectrum of YZFDF in negative ion mode. (c) The chemical structure and $[M + H]^+$ MS analysis spectrum of bilobalide. (d) The chemical structure and $[M + H]^+$ MS analysis spectrum of ginkgolide A. (e) The chemical structure and $[M + H]^+$ MS analysis spectrum of ginsenoside Rg1. (f) The chemical structure and $[M + H]^-$ MS analysis spectrum of cistanoside A. (g) The chemical structure and $[M + H]^+$ MS analysis spectrum of α-asarone.

qPCR RT Kit with SYBR green (TOYOBO, Japan). Sequences of the upstream and downstream PCR primers to detect human Hsp70 mRNA used in qRT-PCR were 5′-GCC ACT CTG CTT ATC AAG TTT C-3′ and 5′-CTC CCA ATG TCG TGT CAA AT-3′, respectively. Upstream and downstream primers for human Grp78 mRNA were 5′-AAA GAA ACC GCT GAG GCT TAT-3′ and 5′-CTG AAA CAG TAT GCC GAC AAG-3′, respectively. Upstream and downstream primers for human 18S rRNA were 5′-CAG CCA CCC GAG ATT GAG CA-3′ and 5′-TAG TAG CGA CGG GCG GTG TG-3′, respectively.

2.8. Statistical Analysis. All data was expressed as the mean ± SEM. Statistical analysis was performed using IBM SPSS Statistics 19. One-way analysis of variance (ANOVA) followed by LSD (Least Significant Difference) test was used to compare the means of three or more normally distributed samples. Student's t-test was used for the evaluation of differences between two groups. Differences were considered to be significant for values of $p < 0.05$.

3. Results

3.1. Structure Identification of Chemical Compounds of the Extracts of YZFDF by HPLC-MC. Most of the main effective compounds of extracts of YZFDF are bilobalides and ginsenosides which exhibited their protonated-molecular ions $[M - H]^+$ in positive ion mode, while the main compounds of Cistanches Herba exhibited their deprotonated-molecular ions $[M + H]^-$ in negative ion mode. As shown in Figure 2, we identified bilobalide and ginkgolide A, the main compounds of *Ginkgo biloba* leaves, and ginsenoside Rg1, the main compound of ginseng, and cistanoside A of Cistanches Herba in the extracts of YZFDF. α-Asarone is a kind of volatile small molecule, which can be detected by using atmospheric pressure chemical ionization (APCI), and the results showed that the main compound of grass leaved sweetflag, α-asarone, existed in this extract of YZFDF.

3.2. YZFDF Can Increase Cell Viability against $A\beta_{1-42}$ Oligomer's Toxicity. To investigate the effects of YZFDF on $A\beta_{1-42}$ oligomer induced neurotoxicity, SH-SY5Y cells were pretreated with or without YZFDF for 2 h and then incubated with 10 μM $A\beta_{1-42}$ oligomer for 24 h. As shown in Figure 3, we can find that cells treated with $A\beta_{1-42}$ oligomer alone showed significant low viability compared to cells in the control group. Furthermore, in cells pretreated with various concentrations of YZFDF, cell viability increased in a dose-dependent manner. Besides, in cells pretreated with 50 μg/mL and 100 μg/mL YZFDF, cell viability was significantly increased in contrast to cells treated with $A\beta_{1-42}$ oligomer alone.

3.3. YZFDF Protects SH-SY5Y Cells from $A\beta_{1-42}$ Oligomer Induced ER-Related Apoptosis. Caspase-12 is a member of apoptosis protein family produced by ER. The malfunction of ER leads to the activation ERS, with a subsequent increased expression of cleaved caspase-12, with the activation of ER-related cell apoptosis in the end. As shown in Figure 4, in cells pretreated with various concentrations of YZFDF,

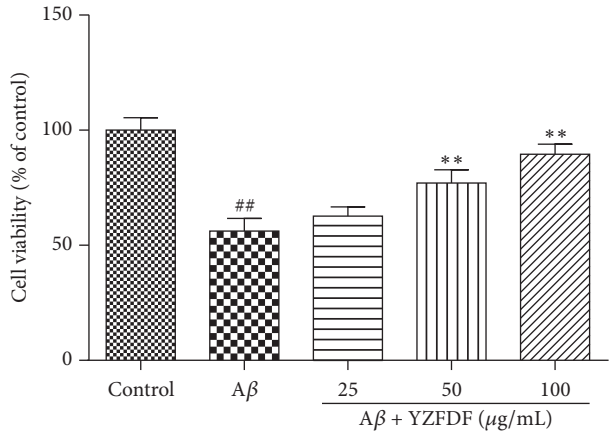
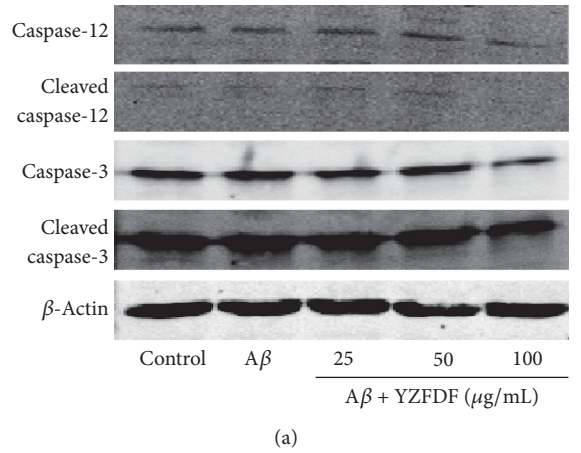
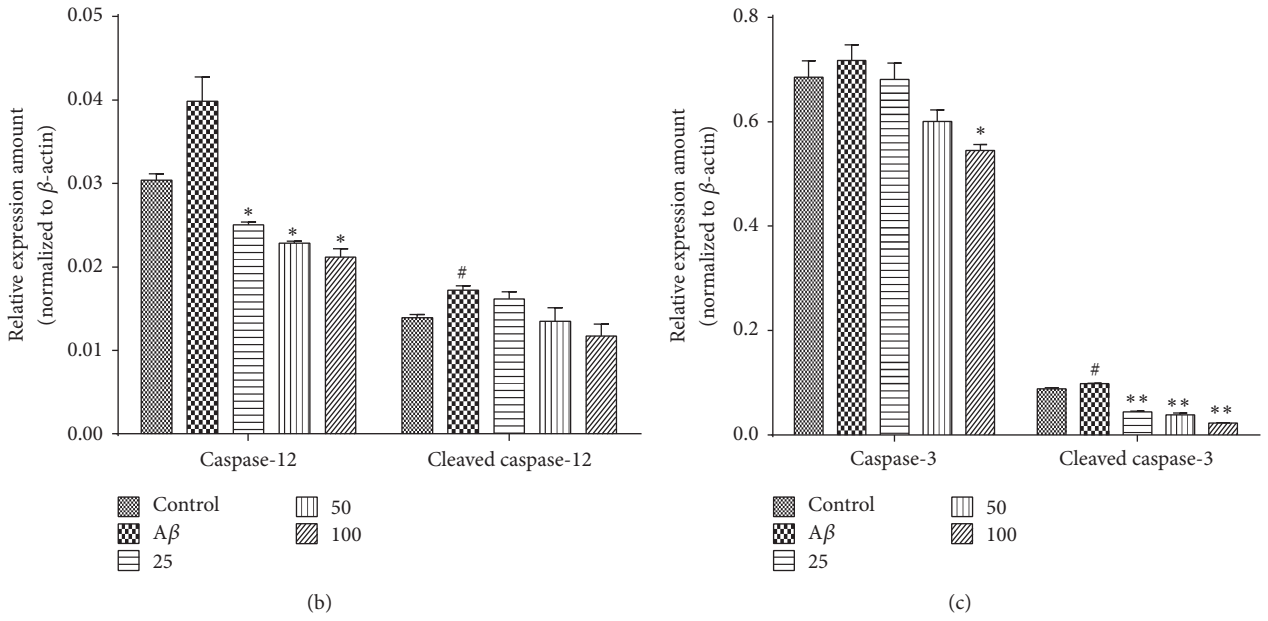


Figure 3: YZFDF increased SH-SY5Y cell viability against $A\beta_{1-42}$ oligomer toxicity. Cells were pretreated with or without various concentrations of YZFDF for 2 h and then incubated with 10 μM $A\beta_{1-42}$ oligomer for 24 h. Subsequently, cell viability was measured by the MTT assay. The results are shown as mean ± SEM ($^{\#\#}p < 0.01$, control versus Aβ; $^{**}p < 0.01$, Aβ + YZFDF versus Aβ).



(a)



(b) (c)

Figure 4: YZFDF protects SH-SY5Y cells from $A\beta_{1-42}$ oligomer induced ER-related apoptosis. YZFDF decreased the expression of caspase-12, caspase-3, cleaved caspase-12, and cleaved caspase-3 in $A\beta_{1-42}$ oligomer treated cells in a dose-dependent manner. The results are shown as mean ± SEM ($^{\#}p < 0.05$, control versus Aβ; $^{*}p < 0.05$, $^{**}p < 0.01$, Aβ + YZFDF versus Aβ).

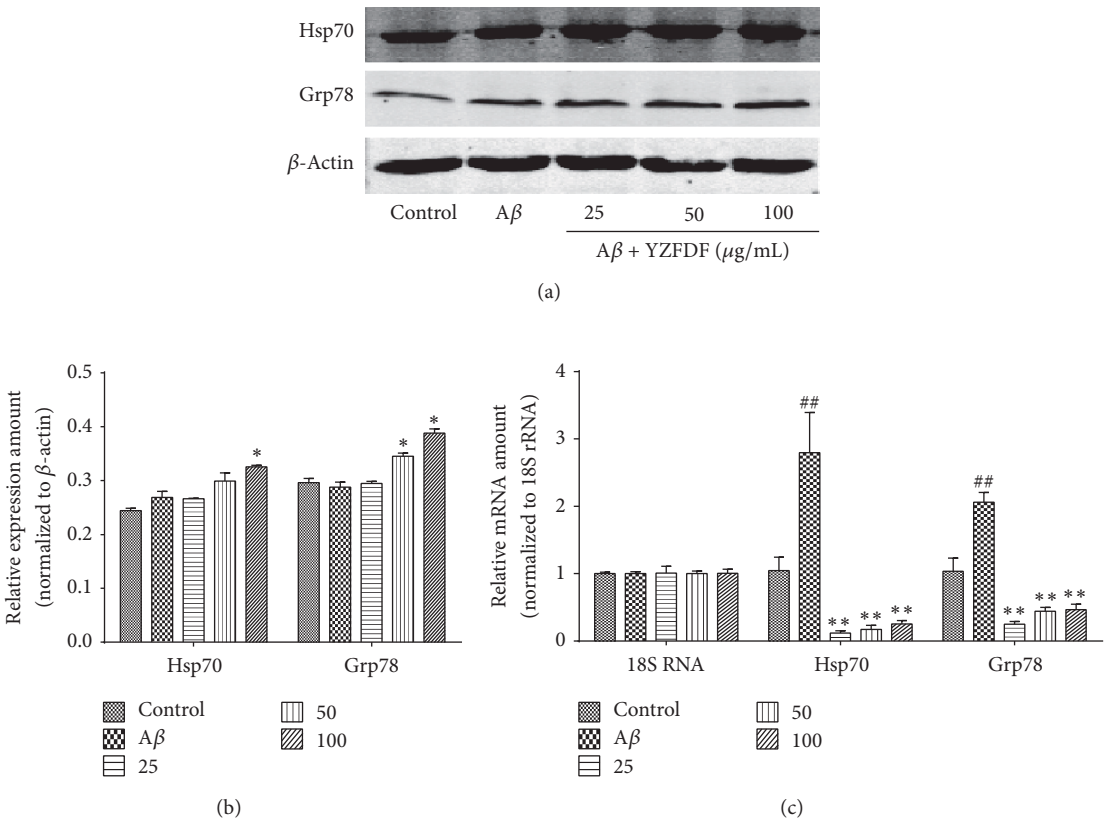


Figure 5: YZFDF influenced the protein and mRNA expression of Hsp70 and Grp78 in SH-SY5Y cells. Cells were pretreated with or without various concentrations of YZFDF for 2 h and then incubated with 10 μM Aβ_{1-42} oligomer for 24 h. (a) and (b) showed that YZFDF increased Hsp70 and Grp78 expression in SH-SY5Y cells in a dose-dependent manner compared to that seen for treatment with Aβ_{1-42} oligomer alone. (c) showed that YZFDF could increase Hsp70 and Grp78 mRNA expression in a dose-dependent manner, while the mRNA expression was lower than that seen for treatment with Aβ_{1-42} oligomer alone. The results are shown as mean ± SEM ($^{\#\#}p < 0.01$, control versus Aβ; $^{*}p < 0.05$, $^{**}p < 0.01$, Aβ + YZFDF versus Aβ).

the expression of caspase-12 and caspase-3 decreased in a dose-dependent manner while Aβ treated alone group showed an increase in expression of caspase-12 and caspase-3. Evidence of caspases activation is provided by the proteolysis of procaspases into smaller cleaved caspases fragments. Thus, we detected the expression of cleaved caspase-12 and caspase-3. Compared to control group, Aβ treated alone group expressed an increased expression of cleaved caspase-12 and cleaved caspase-3, while YZFDF pretreatment restored the increased expression of cleaved caspase-12 and cleaved caspase-3 dose-dependently, especially cleaved caspase-3.

3.4. YZFDF Increased the Expression of Hsp70 and Grp78 in SH-SY5Y Cells. To explore the relationship between ER stress and the neuroprotective effect of YZFDF, we further investigate the effect of YZFDF on the expression of Grp78 (an ER stress marker) and Hsp70. The data showed that, in cells pretreated with YZFDF, the protein expression of Grp78 and Hsp70 is significantly higher than in cells treated with Aβ alone in a dose-dependent manner (Figures 5(a) and 5(b)). However, the PCR results showed that Aβ treated alone group manifested a significant higher mRNA expression of Hsp70 and Grp78 than other groups, while YZFDF pretreated groups still showed a dose-dependent increased mRNA expression of Hsp70 and Grp78 (Figure 5(c)).

3.5. YZFDF Can Activate the Akt in SH-SY5Y Cells. Evidence suggested that Akt pathway is a prosurvival signaling system in neurons [27]. To identify whether the neuroprotective effect of YZFDF is related to the activation of Akt, we further used the western blotting method to explore the expression of Akt1 and pAkt. As shown in Figure 6, we can see that Aβ_{1-42} oligomer decreased the expression of Akt1 and pAkt, while, in cells pretreated with YZFDF, Akt1 and pAkt protein expression increased dose-dependently (Figure 6(b)). Besides, compared to Aβ treated alone group, 50 μg/mL and 100 μg/mL YZFDF pretreated groups showed significant Akt activation (Figure 6(c)).

4. Discussion

In this study, the effective compounds of YZFDF have been identified. We identified the main compounds of *Ginkgo biloba* leaves, bilobalide and ginkgolide A, and found ginsenoside Rg1, the main compound of ginseng, in YZFDF

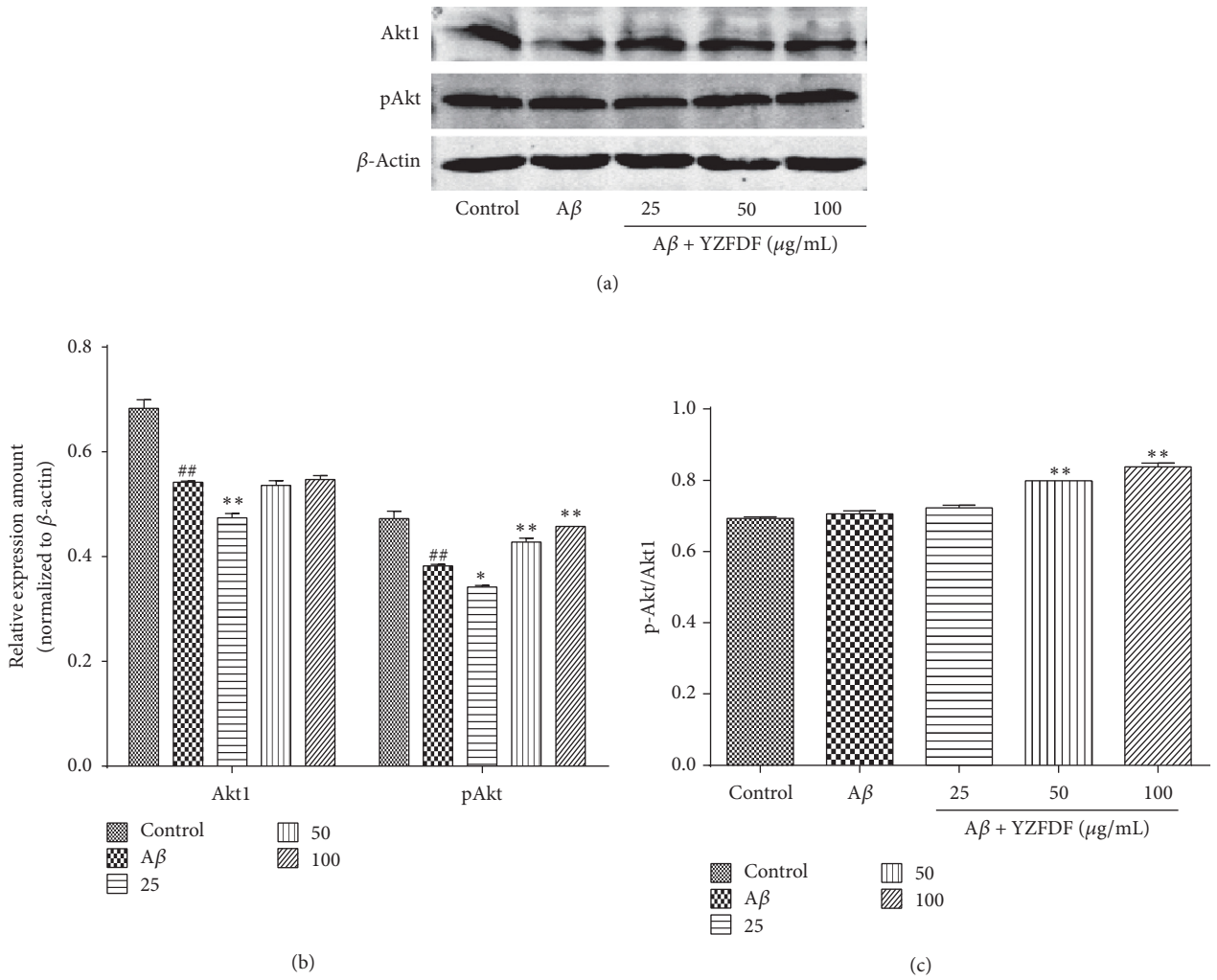


Figure 6: YZFDF activated the Akt expression in a dose-dependent manner. Cells were pretreated with or without various concentrations of YZFDF for 2 h and then incubated with 10 μM Aβ_{1-42} oligomer for 24 h. The data are shown as mean ± SEM ($^{\#\#}P < 0.01$, control versus Aβ; $^{*}P < 0.05$, $^{**}P < 0.01$, Aβ + YZFDF versus Aβ).

samples. Moreover, we also identified cistanoside A of Cistanches Herba and α-asarone of grass leaved sweetflag in YZFDF samples. Our previous studies found that the EGb761, the extract of *Ginkgo biloba* leaves which is an important herb in this formula, exhibited a good protective effect on endothelial cells [23] and neuron cells [22]. In this work, the integral formula also restored SH-SY5Y cell viability from Aβ_{1-42} oligomer induced decreased cell viability (Figure 3).

The "amyloid cascade hypothesis" indicates that the aggregation of Aβ triggers a series of downstream events including the formation of neuritic plaques and neurofibrillary tangles and neuronal loss and ultimately clinical dementia [28, 29]. Available data presents abnormal accumulation of Aβ as a key factor, which can result in mitochondrial and ER dysfunction and eventually cell apoptosis [30]. Caspases have a central role in mammalian cell apoptosis and are classified into two different groups, namely, initiator and effecter caspases. Caspase-12 is an ER-specific initiator caspase, which, later on, activates the effector caspase-3, eventually leading to ER-mediated cell death pathway. Our data showed that YZFDF can not only decrease the expression of procaspase-12 and procaspase-3, but also decrease the expression of activated caspases, cleaved caspase-12, and cleaved caspase-3, while, in Aβ_{1-42} treated alone group, the expression of these apoptosis proteins increased (Figure 4). This indicates that YZFDF can increase neuron cell viability via attenuating ER-related apoptosis and Aβ_{1-42} can induce ER-related apoptosis in SH-SY5Y cells.

Evidence showed that exogenous Aβ could induce ERS and activate mitochondria- and ER-mediated cell apoptosis [31, 32]. As a result of ERS, Grp78, the marker protein of ERS, dissociates from ER domains and binds to overloaded Aβ, to reestablish homeostasis [33], and Hsp70, the homologue of Grp78, plays a key role in maintaining protein homeostasis via participating in helping protein refolding; thus, we further explored the expression of Hsp70 and Grp78. Our data showed that Aβ_{1-42} can significantly increase the mRNA expression of Hsp70 and Grp78, but the protein expression of Hsp70 and Grp78 did not show a significant increase (Figure 5). On the other hand, the western blotting results

showed that YZFDF increased the expression of Hsp70 and Grp78 in SH-SY5Y cells compared to Aβ treated alone group, while the PCR results showed a lower expression, but a dose-dependent increased expression in YZFDF pretreated groups (Figure 5). The lower expression of mRNA and the higher expression of protein of YZFDF pretreated groups compared to Aβ treated alone group gave us a clue that YZFDF can influence ERS and protein folding process to play a role in neuroprotection, but the precise mechanism about the relationship between lower mRNA expression and higher protein expression needs further exploration. What is more, we detected whether the attenuation of ER-mediated apoptosis caused by YZFDF pretreatment had a relationship with Akt activation. Our results showed that YZFDF pretreatment could increase the expression of Akt1 protein and pAkt protein, inducing activation of Akt in a dose-dependent manner, while Aβ_{1-42} treated alone group showed a decreased protein expression of Akt1 and p-Akt (Figure 6); this may reveal a relationship between Akt activation and ER-related apoptosis. All these data showed that YZFDF had a strong neuroprotective effect against Aβ neurotoxicity.

Nowadays, there still exist little effective therapeutic methods to cure AD. With the advantages of multitargets, multipathways, and multicomponents, TCM herbs show a good curative effect with little side effects and should be a candidate for curing neurodegenerative diseases like AD. Our experiential prescription, YZFDF, has shown a strong neuroprotective effect against Aβ through attenuating ER-mediated apoptosis, mediating protein folding process and ERS, and activating Akt expression. This study highlights the potential for YZFDF to be a potent drug candidate for the treatment of AD. However, further studies on this formula like the precise roles of its effective compounds in neuroprotection and the clear mechanism of its neuroprotective effect in restoring the AD pathology still need to be done in the future.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Acknowledgments

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Research Article

Preservation of Cognitive Function by *Lepidium meyenii* (Maca) Is Associated with Improvement of Mitochondrial Activity and Upregulation of Autophagy-Related Proteins in Middle-Aged Mouse Cortex

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Maca has been used as a foodstuff and a traditional medicine in the Andean region for over 2,000 years. Recently the neuroprotective effects of maca also arouse interest of researchers. Decrease in mitochondrial function and decline in autophagy signaling may participate in the process of age-related cognitive decline. This study aimed to investigate if maca could improve cognitive function of middle-aged mice and if this effect was associated with improvement of mitochondrial activity and modulation of autophagy signaling in mouse cortex. Fourteen-month-old male ICR mice received maca powder administered by gavage for five weeks. Maca improved cognitive function, motor coordination, and endurance capacity in middle-aged mice, accompanied by increased mitochondrial respiratory function and upregulation of autophagy-related proteins in cortex. Our findings suggest that maca is a newly defined nutritional plant which can improve mitochondrial function and upregulate autophagy-related proteins and may be an effective functional food for slowing down age-related cognitive decline.

1. Introduction

Lepidium meyenii (maca) has been used as food and a traditional medicine in the Andean region for over 2,000 years [1]. Recently maca has been developed as dietary supplement for its potential advantageous effects on physical and sexual activity [2–4]. Multiple biological functions of maca have been demonstrated by human and animal studies, including enhancing sexual drive and fertility in men and women [2, 3], increasing vigor and energy levels [5], and reducing depression [6, 7].

Recently potential neuroprotective effects of maca have been studied in both *in vitro* and *in vivo* experimental models. A significant concentration-dependent protective effect of maca was observed in H_2O_2-treated crayfish neurons [8]. In another rat stroke model, the pentane extract of maca at lower dose (3 mg/kg per injection; 30 min prior to stroke; and 1 h after stroke) decreased infarct volumes, and higher doses (10 and 30 mg/kg per injection, resp.; 30 min prior to stroke and 1 h after stroke) increased infarct volumes compared to controls [8]. Previous studies have also shown that maca could improve learning and memory in some experimental animal models such as ethanol-, scopolamine-, or ovariectomy-induced memory impairment [9–11]. However, it is still not clear whether maca has some prophylactic effects on the age-related cognitive decline.

Mitochondria play a pivotal role in aging and are closely related to the earlier stages of some events that result in

aging phenotype [12]. With age, changes in mitochondrial morphology and respiratory function could increase the production of reactive oxygen species (ROS) and thus affect cell homeostasis [12]. Mounting evidence indicated that age-dependent increase in mitochondrial dysfunction is involved in brain aging and neurodegenerative diseases [13]. Therefore, mitochondria are increasingly considered to be a target for preventing brain aging. Neurons are highly dependent on mitochondrial respiratory function due to their unique function and bioenergetic requirements [14]. Thus we proposed that maca can preserve cognitive function in middle-aged mice through improving mitochondrial function.

Autophagy, which is a cellular catabolic mechanism essential for degradation of misfolded proteins and dysfunctional organelles, has been implicated in brain aging and multiple neurodegenerative diseases [15]. Lipinski et al. demonstrated that autophagy was transcriptionally downregulated during aging in the human brain [15]. Pharmacological activation of autophagy has been shown to facilitate the clearance of intracellular protein aggregates such as α-synuclein [16] and amyloid β [17, 18] and promote neuronal survival in a series of disease models [19, 20]. It has been reported that diets containing 6% (w/w) walnuts can effectively activate autophagy in the striatum and hippocampus of 19-month-old rats [21] and improve cognitive function [22]. Thereby, we think it is of interest to investigate whether maca has some effects on autophagy signaling, consequently improving the cognitive function of middle-aged mice.

Existing evidence revealed that neuroprotective effects of maca may be related to improvement of antioxidant activity and reduction in oxidative stress [7, 9–11, 23]. Mitochondria are a main source of reactive oxygen species (ROS) and also a target of ROS at the same time. Autophagic turnover of cellular constituents is of great importance, especially in eliminating dysfunctional or damaged mitochondria, thus counteracting degeneration [24]. The purpose of this study was to investigate if the preservation of cognitive function by maca in middle-aged mice was associated with improvement of mitochondrial activity and modulation of autophagy signaling.

2. Materials and Methods

2.1. Plant Materials and Chemical Properties. Maca powder was imported from Peru and was a gift from Nanjing Bio-Array Technology Company (Nanjing, China) and was chemically identified by Suzhou Institute of Chinese Materia Medica, Suzhou University. The macamides have been considered to be the signature compounds of maca [25] and have shown promising pharmacological activities in some studies [26, 27]. Thus the macamides were measured by high-performance liquid chromatography (HPLC) using standard maca product from Peru as a reference. The results proved that maca powder used in this study has similar levels of active compounds as the standard (Figure 1).

The chemical properties of maca powder used were shown in Table 1.

Table 1: Chemical composition of maca powder.

Number	Specification	Unit per 100 g of product	Maca powder
1	Energy value	kJ	1313
2	Carbohydrates	g	46.1
3	Crude protein	g	21.9
4	Fat	g	0.9
5	Dietary fiber	g	15.6

2.2. Animals and Treatment. Fourteen middle-aged (14-month-old) male ICR mice (46.1 ± 5.1 g) were obtained from the Experimental Animal Center of Soochow University. One week after arriving at the facility, mice were randomly assigned to two groups: (1) control group (CON, $n = 7$), (2) maca-treated group (MACA, $n = 7$). Maca powder (150 meshes) was suspended in saline at 50 mg/mL. The mice in MACA received maca powder suspension (0.1 mL/10 g body weight, 500 mg maca powder/kg body weight) administered by gavage once a day for 5 weeks. The mice in CON received equal volumes of saline with the same method. All mice were kept in individual cages with standard food and water *ad libitum* in a temperature (22° ± 2.5°C) and light-controlled (12 : 12 h light-dark cycle) environment. The body weights of mice were recorded every week (Figure 2). The study protocols were approved by Animal Care Ethical Committee of Soochow University. The experiment design was shown in Figure 3.

2.3. Behavioral Tests

2.3.1. Morris Water Maze Test. Morris water maze (MWM) test was similar to those described in a previous study [28]. Briefly, all mice were tested for spatial learning and memory performance with the Morris water maze. The maze consisted of a 1.2 m diameter circular white fiberglass pool filled to a depth of 50 cm with water (26 ± 1°C) made opaque with the addition of nontoxic white latex paint and was in a room with extra maze cues on the walls around the pool. A circular escape platform (10 cm diameter) was submerged approximately 1 cm below the water surface in one of the quadrants of the pool, and this position remained constant throughout testing. All mice were first habituated to the maze with a 60 s free-swim in the pool prior to testing. Mice then completed 24 trials over 6 consecutive days to learn the location of the submerged platform (the spatial acquisition phase, four trials per day; 60 s maximum trial duration). If the mice did not find the platform within 60 seconds, it was placed on the platform for 15 seconds. Latencies to locate the hidden platform were monitored by a video camera mounted in the ceiling and a computerized tracking system (ANY-maze video tracking system, Stoelting Co.). On day 7, the mice were given a 60 s retention test of the spatial location (probe test) with the platform absent, and the times of crossing the previous hidden platform were recorded.

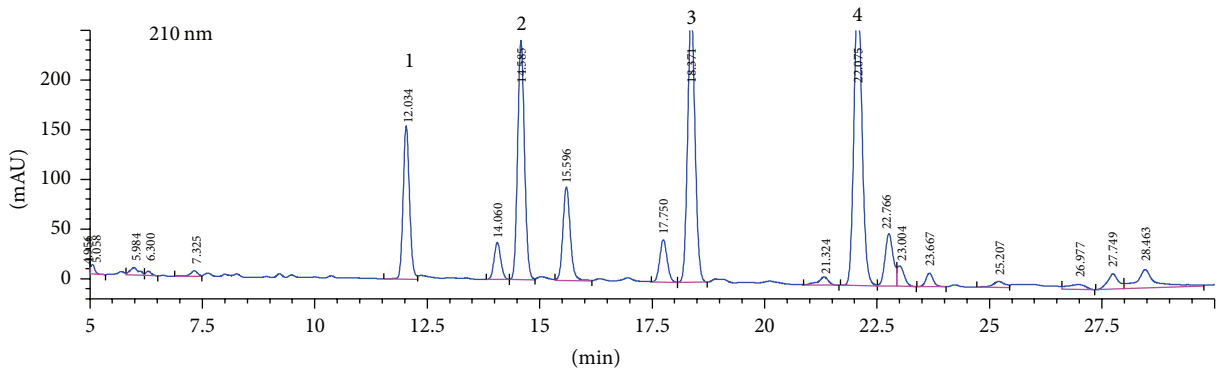

Figure 1: Analysis of macamides in maca powder measured by HPLC using standard maca product from Peru as a reference. (1–4): macamides.

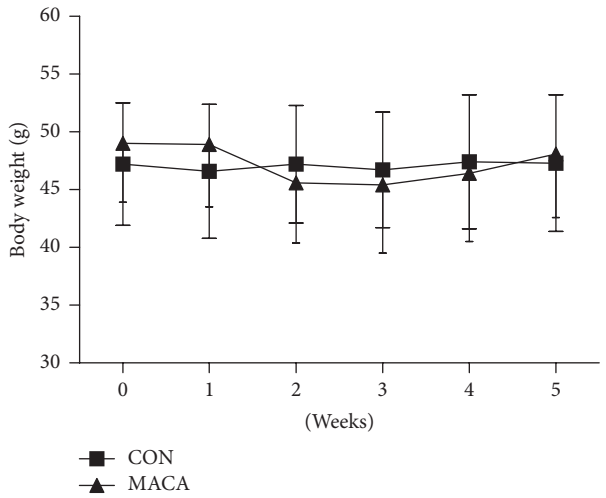

Figure 2: The effects of maca on body weight of middle-aged mice.

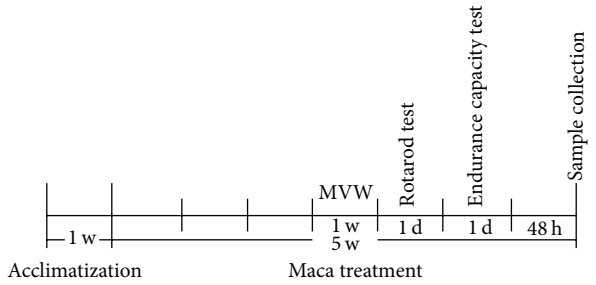

Figure 3: Timeline for experimental design. The mice were allowed to acclimatize to the environment for the first week. Then the mice in MACA were treated with maca powder suspension administered by gavage once a day for 5 weeks. From the 25th day of maca treatment, all mice underwent Morris water maze test (1 w), rotarod test (1 d), and endurance capacity test (1 d) in succession. Sample collection was performed 48 h after the endurance capacity test to minimize the influence of behavioral tests.

2.3.2. Rotarod Assessment of Motor Coordination. Then motor coordination was assessed with a rotarod treadmill (San Diego Instruments, San Diego, CA, USA) according to a previous study [29] with minor modifications. Mice were allowed to acclimate to the rod at fixed speed (5 rpm/min) for 30 s. Then the mice were tested using the accelerated version of the rotarod test, in which the rotating speed was accelerated from 5 rpm/min to 50 rpm/min within 5 min. The fall-off latency was averaged from three tests.

2.3.3. Measurement of Swimming Endurance Capacity. Swimming endurance capacity was assessed according to a previous study [30] with minor modifications. All mice were subjected to a progressive load test in the swimming apparatus (60 cm × 55 cm × 80 cm) in order to determine endurance capacity. The mice were placed in the water (26 ± 1°C, 45 cm in depth) with a load (lead wire) attached to the tail corresponding to 1% of their body weight, with a step increase in weight (1% of each mouse's body weight) every 3 min until exhaustion (determined by 10 continuous seconds submerged). The time to exhaustion was recorded as the endurance capacity of each mouse.

2.4. Tissue Harvesting. Forty-eight h after endurance capacity test, the mice were sacrificed by decapitation under anesthesia and the cortex were dissected and stored at −80°C prior to analysis.

2.5. Immunoblotting. Frozen cortical tissues were lysed and used for immunoblotting as described previously [31]. Equal amounts (30–45 μg) of total protein extracts were separated by 10–15% SDS-PAGE and the separated proteins were transferred onto nitrocellulose membranes. Nonspecific binding was blocked by incubating membranes in Tris buffered saline containing 0.05% Tween 20 (v/v) and 5% nonfat milk (v/v) for 1 h. Blots were incubated with primary antibodies as follows: LC3 A/B (1 : 1000; Abcam), Atg7 (1 : 1000; Beyotime Institute of Biotechnology), Beclin1 (1 : 1000, Cell Signaling), Total OXPHOS Rodent WB Antibody Cocktail (1 : 250, Abcam), and β-actin (1 : 5000; Sigma) at 4°C overnight. The membranes were washed and incubated with IRDye secondary antibodies (1 : 10,000; Li-Cor Bioscience) for 1 h at room temperature. The images of protein-antibody interaction were captured with the Odyssey infrared imaging system (Li-Cor Bioscience) and analyzed with Image J with normalization to the loading control β-actin.

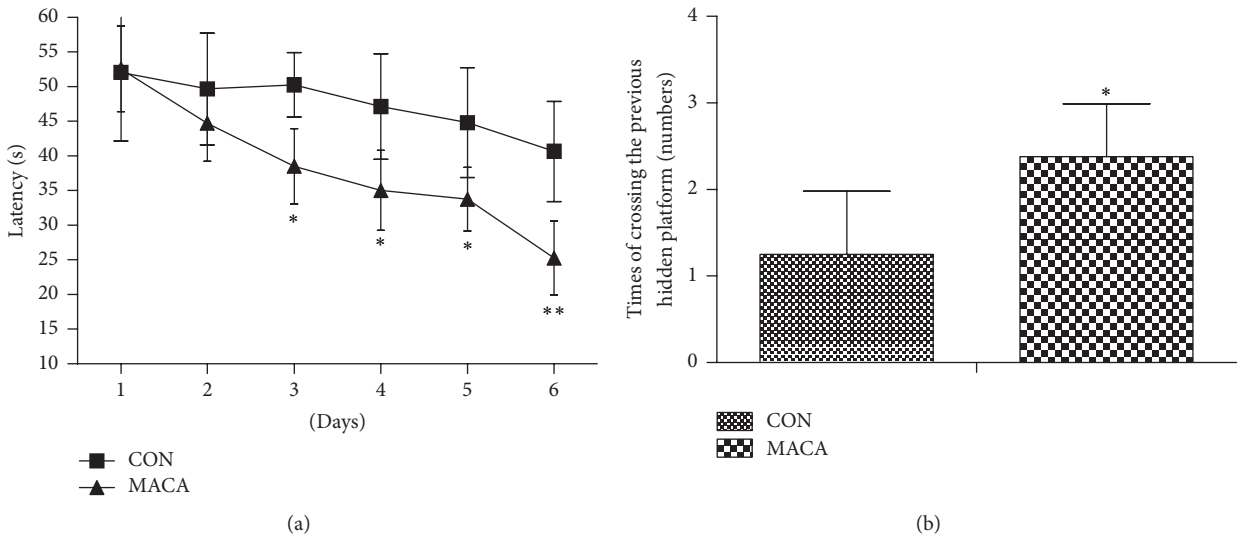

Figure 4: Maca improves spatial learning and memory of middle-aged mice. Values are the mean ± SD. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus CON.

2.6. Statistical Analysis. For the MWM test, escape latencies were analyzed with repeated measures analysis of variance (ANOVA). One-way ANOVA and Newman-Keuls post hoc tests (two-tailed) were performed to determine the differences of escape latencies between groups at different time point and the differences of times of crossing the previous hidden platform between groups in the probe test. For the Western blot analysis and rotarod test, nonparametric Mann-Whitney *U* test (two-tailed) was used. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Maca Improved Spatial Learning and Memory in Middle-Aged Mice. Learning and memory capacity was assessed in all mice using the Morris water maze test. Mice were tested in the hidden platform version of the water maze for 6 consecutive days, and goal latencies were evaluated. During the spatial acquisition phase, mice in MACA spent less time in locating the hidden platform than CON mice on days 3, 4, 5, and 6, respectively (Figure 4). A probe trial test was performed on day 7. Mice in maca-treated group showed an increase of times of crossing the previous hidden platform compared with mice in CON, suggesting an improvement in memory retention (Figure 4).

3.2. Maca Improved Motor Coordination and Swimming Endurance Capacity in Middle-Aged Mice. Motor coordination was assessed after five weeks of maca treatment with a rotarod treadmill. An increase in latency to fall in maca-treated mice was shown in Figure 5. This finding suggests an improved motor coordination ability of mice after maca treatment.

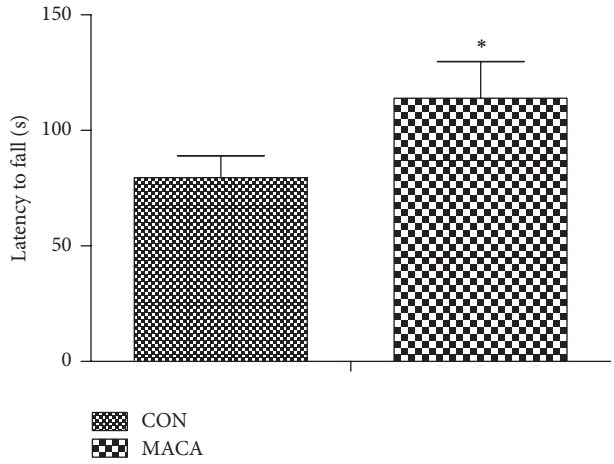

Figure 5: Maca improves motor coordination of middle-aged mice. Values are the mean ± SD. $^{*}p < 0.05$ versus CON.

The swimming time to exhaustion was measured to investigate if maca had the antifatigue property in middle-aged mice. As shown in Figure 6, the swimming time of the mice in MACA was significantly higher than that in CON.

3.3. Maca Increased the Expression of Subunits of Mitochondrial Respiratory Chain Complex in the Cortex of Middle-Aged Mice. The protein levels of oxidative phosphorylation (OXPHOS) enzyme complexes have been used as indicator of mitochondrial metabolic function in previous studies [32–37]. As shown in Figure 7, there were significant increases in OXPHOS I, II, III, IV, and V complexes in the cortex of

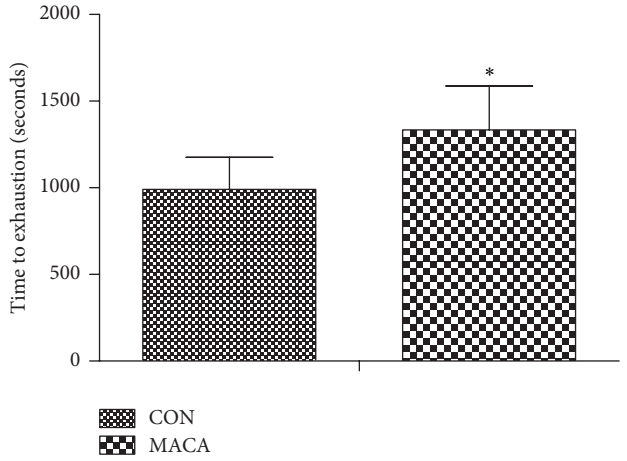

Figure 6: Maca improves endurance capacity in middle-aged mice. All mice were subject to a progressive load test in the swimming apparatus to determine the endurance capacity. Values are the mean ± SD. $^{*}p < 0.05$ versus CON.

maca-treated middle-aged mice, when compared to the age-matched controls, suggesting an improvement of mitochondrial respiratory function in the cortex of maca-treated mice.

3.4. Maca Upregulated the Expression of Autophagy-Related Proteins in Cortex of Middle-Aged Mice. Next we measured levels of LC3, Atg7, and Beclin1 to determine the effects of maca on autophagy-related proteins in cortex. The protein level of LC3-II and the ratio LC3-II/LC3-I were significantly higher in MACA than those in CON (Figure 8(a)). In addition, Atg7 and Beclin1 protein levels were also significantly higher in MACA than those in CON (Figures 8(b) and 8(c)). These data suggest that maca might activate autophagy signaling in cortex of middle-aged mice.

4. Discussion

In the present study, we demonstrated that five weeks of maca supplementation improved cognitive function in middle-aged mice. Besides, maca increased the protein levels of subunits of OXPHOS complexes and autophagy-related proteins in mouse cortex. These data suggest the improvement of cognitive function by maca may be associated with, at least partially, improvement of mitochondrial respiratory function and upregulation of autophagy-related proteins in cortex of middle-aged mouse.

Maca has been demonstrated to possess multiple biological properties, such as antifatigue, improving sexual performance and neuroprotective activities [3, 8, 27, 38]. In this study, supplementation of maca for five weeks significantly improved the endurance capacity and motor coordination in middle-aged mice, which was in accordance with previous studies [3, 38, 39]. Rubio et al. reported that aqueous and hydroalcoholic extracts of black maca improved memory deficits in mice induced by ethanol, scopolamine, or ovariectomy, respectively [9–11]. However, it is still not clear whether maca can improve learning and memory capacity in the middle-aged mice. In this study, the mice in MACA showed better learning and memory ability as assessed by the Morris water maze test, suggesting the potential of maca for preventing cognitive decline in the elderly.

Alterations of mitochondrial functions are linked to brain aging and a few neurodegenerative diseases. With age, mitochondria become progressively inefficient and generate more ROS, which will damage the macromolecules such as lipids, proteins, and carbohydrates [12]. Besides, damaged mitochondria can potentially trigger apoptosis and necrosis and thus lead to cell death [24]. Previous studies have demonstrated that some of the biological actions of maca, including improving endurance capacity and antifatigue property, were associated with the improvement of energy metabolism and antioxidant status [38]. In addition, aqueous extract of black maca has been reported to improve experimental memory impairment induced by ovariectomy via downregulation of oxidative stress [11]. Our results demonstrated for the first time that the neuroprotective effects of maca were accompanied by an improvement of mitochondrial respiratory function. Nevertheless, further studies are required to clarify the cause and effect relationship between reduction of oxidative stress and improvement of mitochondrial function by maca.

Dysregulation of autophagy has been considered to be involved in brain aging and multiple neurodegenerative diseases [15]. Moreover, autophagy/mitophagy plays a pivotal role in mitochondrial quality control by eliminating damaged or dysfunctional mitochondria [40]. Our present study showed that maca increased the protein level of LC3-II and the ratio of LC3-II/LC3-I, along with upregulation of Atg 7 and Beclin1 proteins, indicating autophagy signaling might be activated in the cortex of maca-treated middle-aged mice. Thus, the restoration of cognitive function in middle-aged mice by maca might also be associated with upregulation of autophagy-related proteins.

In summary, the present study demonstrated for the first time that maca improves cognitive function in middle-aged mice, and this effect may be associated with improved mitochondrial respiratory function and upregulation of autophagy-related proteins.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper and regarding the funding that they have received.

Authors' Contributions

Shan-Shan Guo and Xiao-Fang Gao contributed equally to this work.

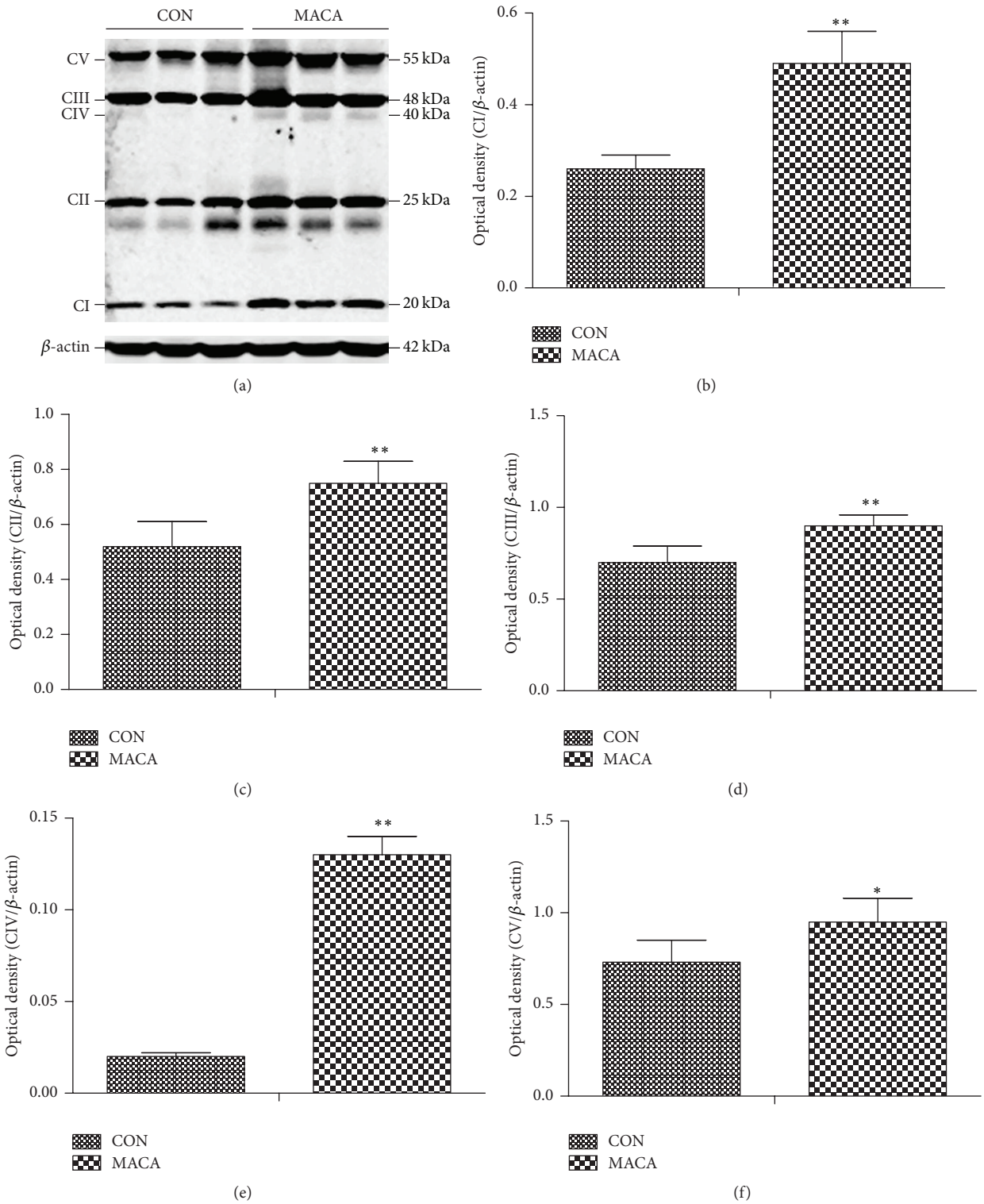

Figure 7: Maca increases the protein expression of subunits of mitochondrial respiratory chain complexes in cortex of middle-aged mice. The cortex was extracted from different groups of mice and subjected to Western blot analysis of OXPHOS. Representative immunoblot images (a) and quantification of mitochondrial OXPHOS complexes I–IV (b–f) in the hippocampal extracts of mice. Values are the mean ± SD. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus CON.

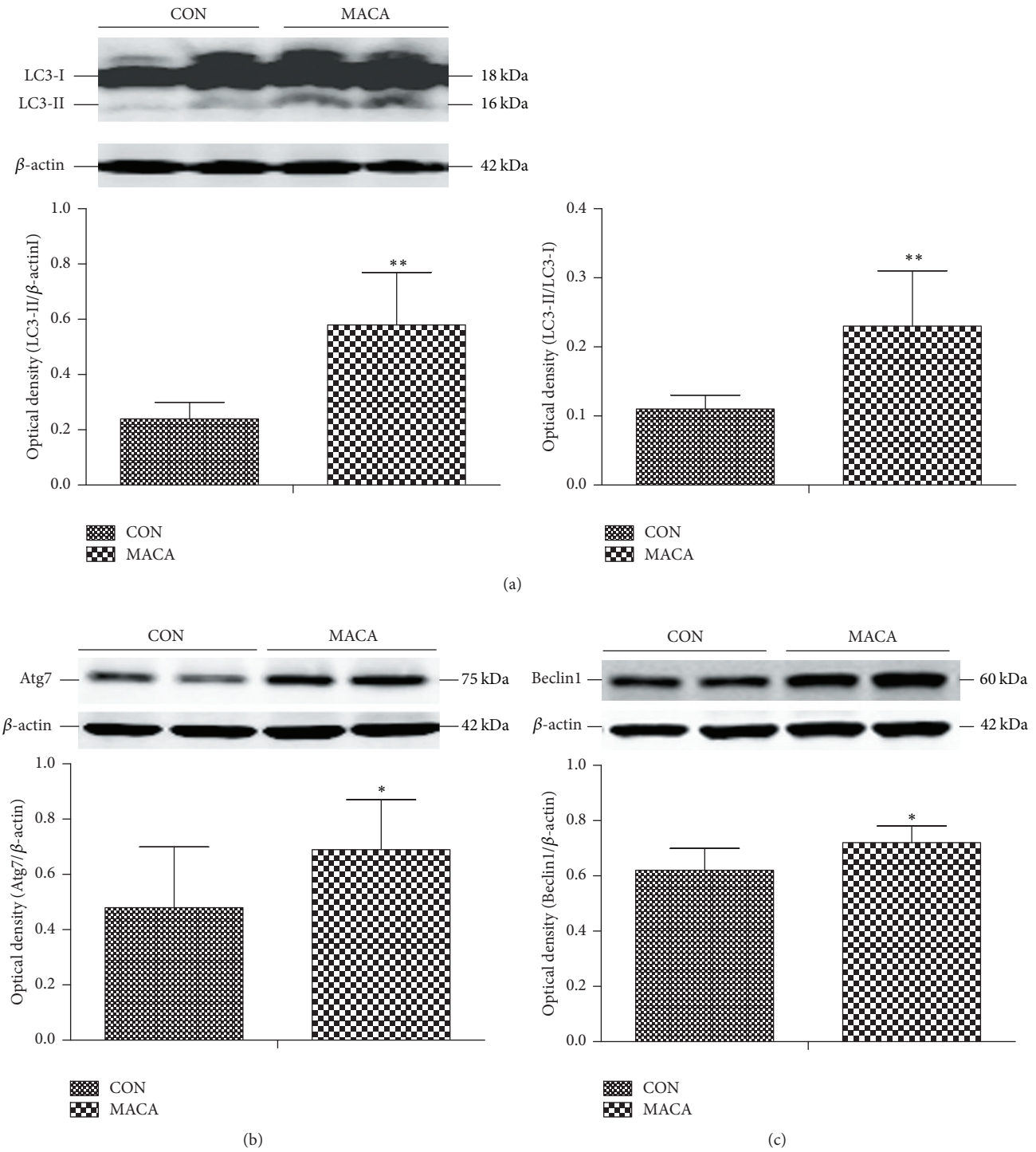

Figure 8: Maca upregulates autophagy-related proteins in cortex of middle-aged mice. The cortex was extracted from different groups of mice and subjected to Western blot analysis of LC3, Atg7, and Beclin1. (a) LC3; (b) Atg7; (c) Beclin1. Values are the mean ± SD. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus CON.

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