HERBAL MEDICINES IN MANAGING STROKE AND NEURODEGENERATIVE DISEASES – IS THERE EVIDENCE BASED ON BASIC AND CLINICAL STUDIES?

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HERBAL MEDICINES IN MANAGING STROKE AND NEURODEGENERATIVE DISEASES – IS THERE EVIDENCE BASED ON BASIC AND CLINICAL STUDIES?

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EDITORIAL
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Editorial: Herbal Medicines in Managing Stroke and Neurodegenerative Diseases—Is There Evidence Based on Basic and Clinical Studies?

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Keywords: herbal medicine, ethnopharmacology, plant derivatives, molecular mechanism, stroke, neurodegenerative disorders

Editorial on the Research Topic

Herbal Medicines in Managing Stroke and Neurodegenerative Diseases—is There Evidence Based on Basic and Clinical Studies?

Stroke and dementia are common diseases afflicting the elderly population. Stroke is the leading cause of death in China (Zhou et al., 2016), and Alzheimer's disease (AD) is estimated to be the 2nd economic burden by 2020. Ischemic stroke has been successfully managed with thrombolytic agents (Hacke et al., 1995; Tsivgoulis et al., 2018) and endovascular thrombectomy (Zi et al., 2021; Suzuki et al., 2021), but a decent proportion of patients still cannot completely restore their neurological functions. These patients with remaining symptoms will be the potential target population of herbal recipes as an adjuvant therapy.

In searching for potential therapeutics that could serve as adjuvant therapies for patients suffering from these diseases, it has been revealed that some herbal recipes including Chinese herbs and other traditional medicines have been widely used by TCM practitioners and physicians in other countries (Iwasaki et al., 2004; Fu et al., 2013; Wang et al., 2015). For the same clinical manifestations, different herbal recipes can be used based on the diagnosis by individual TCM practitioners. There must be is a significant difference in the etiology and pathogenic mechanisms of these diseases. Though there is a large number of publications on clinical use of herbal recipes, few of them comply with the criteria of randomized controlled trials (Fu et al., 2013). It is, therefore, necessary to assess the scientific evidence for these herbal recipes in managing the above-mentioned illnesses using state of the art pharmacological techniques. This will not only facilitate the discovery of new therapeutics or compounds, but also refresh our knowledge in understanding how these diseases develop.

This Research Topic is a collection of nine articles, including four original articles, four review articles and one clinical trial protocol, aiming to examine the commonly used herbal recipes and the molecular mechanisms underlying their therapeutic effects, especially on neurodegenerative diseases.

Stroke is the second leading cause of death worldwide and the first in China. Though intravenous thrombolysis and endovascular thrombectomy have significantly improved the outcome of patients with acute ischemic stroke, a decent proportion of patients were ineligible for these therapies or disabled even after receiving these treatments. New therapies are needed to manage these patients.

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Feng et al. reported that Panax notoginseng saponins (PNS) (Xueshuantong lyophilized powder), an extract from the roots and rhizomes of Panax notoginseng, were shown to increase brain perfusion and neural plasticity through anti-inflammatory, antioxidant, and anti-apoptosis mechanisms. However, the therapeutic effect of PNS has not been confirmed in large-scaled randomized clinical trials. They aim to conduct an RCT by recruiting 480 patients with acute ischemic stroke. Their protocol was submitted to this journal. In a review, Wang et al. summarized evidence of ginseng Rb1, one of the five effective components of PNS, in managing ischemic stroke. It was found that ginseng Rb1 exhibited its protective effect through antioxidant, anti-inflammatory, anti-apoptosis capacities. In addition, it also suppressed excitotoxicity and calcium influx, maintained the integrity of the blood-brain barrier and energy metabolism. Zhang et al. explored the protective effect and the underlying mechanism of An-gong-niu-huang wan pre-treatment on cerebral ischemia and found that it mitigated ischemic injury by reversing the up-regulation of reactive oxygen species and malondialdehyde as well as increasing the expression of p-GSK-3β(Ser9)/GSK-3β (glycogen synthase kinase-3β) ratio and heme oxygenase-1. Yu et al. investigated the synergistic effect of Ligusticum chuanxiong Hort and borneol. It was found that Ligusticum chuanxiong Hort promoted neurogenesis and preservation of mature neurons, whereas borneol improved the ultrastructure of the blood brain barrier and increased expression of tight junction associated proteins, vascular endothelial growth factor, and vascular endothelial growth factor receptor 2, providing an optimal environment for neurogenesis.

AD is the leading neurodegenerative disease inflicting nearly 43.8 million people in the world. Numerous studies have investigated the mechanisms underlying AD but few drugs have been found due to the complex nature of this disease (Abeysinghe et al., 2020). Endeavoring to isolate effective compounds, Wei et al. tested whether the main active fraction combination (LW-AFC) extracted from Liuweidihuang decoction (LW) can improve cognitive and emotional functions in a cranial-irradiation mouse model. It was found that LW-AFC improved both cognitive and depressive behaviours by increasing the number of neural stem cells in the dorsal hippocampus and rectified the altered microenvironment by increasing the contents of glutathione and other factors. In a meta-analysis, Kwon and Lee reported their findings from 52 studies, including 36 RCTs, on the therapeutic effect of herbal medicine on behavioural and psychological symptoms of dementia (BPSD). Though the level of evidence is not optimal, herbal medicine does serve as a promising therapy complementing the conventional western treatments.

Parkinson's disease is the second leading cause of neurodegenerative disease, severely impacting patients' movement and daily life. Due to limited efficacy of currently available medication and their side-effects, many patients prefer to take herbal medicines. Lin et al. analyzed the usage of Chinese herbal products (CHPs) in the general population by reviewing the National Health Insurance Research Database of Taiwan. They found that the most commonly used formula was Chaihu-Jia-Longgu-Muli-Tang, and *Uncaria tomentosa* is the most widely used herb which has been used to treat non-motor symptoms for PD patients. In understanding the pathogenic mechanism of PD and its treatment, Wu et al. summarized indirect evidence of ferroptosis involved in PD pathogenesis and TCM recipes for managing PD. Puerarin, isolated from a plant and a natural ferroptosis inhibitor, showed potential as a new drug candidate for managing PD. This warrants further studies. Su et al. reviewed experimental evidence of resveratrol's therapeutic mechanisms in animal models. It was found that resveratrol exerted protective effects on mitochondrial and motor functions through its anti-oxidative, anti-inflammatory, and anti-apoptosis capacities.

It is our hope that the articles included in this Research Topic provide an update on therapeutic effects, molecular mechanisms, potential active components, as well as clinical evidence and possible future directions for using herbal medicines for the management of neurodegenerative diseases.

AUTHOR CONTRIBUTIONS

HX, HZ, CL, and HL have been serving as guest editors in collecting articles for the research topic.

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Chinese Herbal Products for Non-Motor Symptoms of Parkinson's Disease in Taiwan: A Population-Based Study

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Objective: Combinations of Chinese herbal products (CHPs) are widely used for Parkinson's disease (PD) in Taiwan. Thereby, we investigated the use of CHPs in patients with PD.

Methods: This study was a population-based cohort study that analyzed the data of patients with PD from the National Health Insurance Research Database. A total of 9,117 patients were selected from a random sample of one million individuals included in this database. We used multiple logistic regression models to estimate the adjusted odds ratios of the demographic factors and analyzed the formula and single CHPs commonly used for PD.

Results: Traditional Chinese medicine users were more commonly female, younger, of white-collar status, and residents of Central Taiwan. Chaihu-Jia-Longgu-Muli-Tang was the most commonly used formula, followed by Ma-Zi-Ren-Wan and then Shao-Yao-Gan-Cao-Tang. The most commonly used single herb was *Uncaria tomentosa* (Willd. ex Schult.) DC., followed by *Gastrodia elata* Blume and then *Radix et Rhizoma Rhei* (*Rheum palmatum* L., *Rheum tanguticum* Maxim. ex Balf., *and Rheum officinale* Baill.). Chaihu-Jia-Longgu-Muli-Tang and *U. tomentosa* (Willd. ex Schult.) DC. have shown neuroprotective

Abbreviations: ARE, anti-oxidant response element; CAM, complementary and alternative medicine; CHP, Chinese herbal products; CI, confidence interval; CJLMT, Chaihu-Jia-Longgu-Muli-Tang; FosB, FBJ murine osteosarcoma viral oncogene homolog B; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase; LHID, longitudinal health insurance database; MZRW, Ma-Zi-Ren-Wan; NHI, National Health Insurance; NHIRD, National Health Insurance Research Database; NMS, non-motor symptoms; NO, nitric oxide; Nrf2, nuclear factor erythroid 2-related factor 2; OR, odds ratio; PD, Parkinson's disease; pERK, phosphorylated extracellular regulated protein kinases; PWS, Ping-Wei-San; ROS, reactive oxygen species; SYGCT, Shao-Yao-Gan-Cao-Tang; TCM, traditional Chinese medicine; TWBXD, Tian-Wang-Bu-Xin-Dan.

effects in previous studies, and they have been used for managing non-motor symptoms of PD.

Conclusion: Chaihu-Jia-Longgu-Muli-Tang and *U. tomentosa* (Willd. ex Schult.) DC. are the most commonly used CHPs for PD in Taiwan. Our results revealed the preferences in medication prescriptions for PD. Further studies are warranted to determine the effectiveness of these CHPs for ameliorating the various symptoms of PD, their adverse effects, and the mechanisms underlying their associated neuroprotective effects.

Keywords: Chaihu-Jia-Longgu-Muli-Tang, Chinese herbal products, Chinese medicine, Parkinson's disease, *Uncaria tomentosa (Willd. ex Schult.) DC.*

INTRODUCTION

Parkinson's disease (PD) results primarily from the loss of dopaminergic neurons in the substantia nigra. Treatment for PD includes drugs such as monoamine oxidase type B inhibitors, dopamine agonists, and levodopa. These agents can increase dopaminergic effects but can also result in side-effects, such as nausea, somnolence, dizziness, and headaches. More serious adverse reactions are common in the elderly, including confusion, hallucinations, delusions, agitation, psychosis, and orthostatic hypotension (Spindler and Tarsy, 2019). Levodopa is the most commonly used treatment for PD and is usually co-administered with an aromatic L-amino acid decarboxylase inhibitor to increase bioactivity, since levodopa is rapidly metabolized to dopamine by peripheral aromatic L-amino acid decarboxylase and catechol-O-methyltransferase (Rocha et al., 2017). However, it cannot penetrate the blood–brain barrier to act on the substantia nigra. Therefore, studies are currently seeking to elucidate methods to increase the levodopa levels in the brain, inhibit peripheral levodopa metabolism, and minimize the prevalence of side-effects (Ferreira et al., 2015).

Due to the limitations and side-effects of conventional therapy, patients often seek complementary and alternative medicine (CAM) (Han et al., 2017). In recent decades, the use of CAM for various diseases has increased among all adult age groups, including the elderly (Clarke et al., 2015). Previous studies have estimated the prevalence of CAM use for PD to be 25.7–76%, with survey response rates of 81–100%. Frequently utilized forms of CAM included acupuncture, massage, herbs, and vitamins/health supplements; these therapies were mainly used to improve the motor symptoms of PD (Wang et al., 2013b). Research has shown that Chinese herbal products (CHPs) have a high efficacy against PD, both in clinical trials and animal studies (Han et al., 2017). Despite the mostly positive attitude of the public toward traditional Chinese medicine (TCM), its clinical indications for specific diseases remain controversial (Hasan et al., 2010; Medagama and Bandara, 2014; Setty and Sigal, 2005). Research on the therapeutic effects of CAM may currently be restricted by small sample sizes or the lack of well-designed randomized controlled trials (Han et al., 2017).

The National Health Insurance (NHI) Research Database (NHIRD), which is widely used in Taiwan, provides a platform for understanding the utilization of CHPs by licensed TCM doctors. In this study, we analyzed the NHIRD to determine CHP utilization patterns for PD. The results may provide valuable information regarding the pattern of CHP prescription by TCM physicians, thereby improving PD treatment and forming the basis for subsequent pharmacological studies.

METHODS

Data Source

The NHI was established on March 1, 1995, and provides medical health care to 23 million residents in Taiwan. The coverage of NHI reached 99% by the end of 2014 and therefore represents most of the medication prescription patterns in Taiwan. Each individual has a unique personal identification number in this database.

For this study, data were extracted from the Longitudinal Health Insurance Database (LHID) 2000, a subset of the NHIRD that contains all medical reimbursement claims made under the NHI program for one million enrollees from 2000 to 2011. The NHI reported that there were no significant biases in sex or age distribution in the LHID 2000 recruitment. The data used were provided by the Taiwan National Health Research Institute and authorized by the Ministry of Health and Welfare, which manages the NHI claims. These are the most recent data available. The study was approved by the Institutional Review Board of China Medical University and Hospital (CMUH104-REC2-115).

Study Subjects

From the random sample of one million individuals enrolled in the LHID 2000, we extracted the data of patients diagnosed with PD (ICD-9-CM332, based on the International Classification of Diseases, ninth revision, clinical modification (ICD-9-CM), between 2000 and 2011. All patients with PD utilized the outpatient services at least twice or inpatient hospitalization at least once, according to the database claims (n = 12,675). We excluded patients with missing data for sex and age (n = 4), patients with existing PD diagnoses before the end of 1999 (n = 3,483), and patients aged ≤20 years (n = 71). There were 9,117 new PD cases, including TCM (n = 4,060) and non-TCM users (n = 5,057). TCM users were those who visited a TCM clinic at least once during the study period. Non-TCM users were those who had never visited a TCM clinic after the initial PD diagnosis (**Figure 1**).

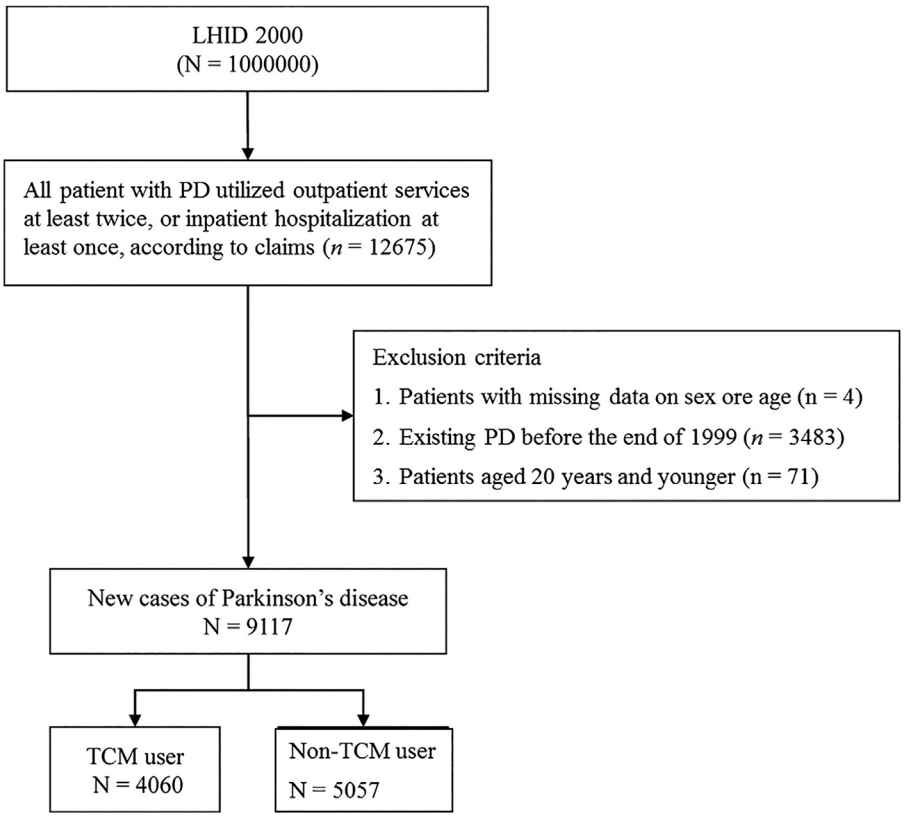

FIGURE 1 | Flowchart of recruitment of subjects from LHID 2000 during 2000–2011 in Taiwan. Abbreviation: TCM, traditional Chinese medicine.

The NHI reimburses two types of Chinese herbal remedies: Chinese single herbs and Chinese herbal formulae. Each Chinese herbal formula is a combination of two or more Chinese single herbs in strict proportions, as per the TCM literature. We analyzed the prescription patterns for PD according to these two types of herbal remedies and their usage rates.

The following sociodemographic data were extracted from the NHIRD: sex, age, occupational status, and residential area. Occupational status was divided into three categories: office workers (white collar), manual workers (blue collar), and others. The residential areas of the population were based on the district branches of the National Health Insurance Administration, consisting of Northern Taiwan, Central Taiwan, Southern Taiwan, Eastern Taiwan, and offshore islands.

Study Analysis

TCM user and non-TCM user data (including age, sex, occupational status, and residential areas) are described as means and standard deviations. Odds ratios (ORs) and 95% confidence intervals (CIs) were used to determine which group might have a greater tendency to use TCM, based on multiple logistic regression models. We used adjusted ORs to address potential sources of bias in the use of TCM. We set statistical significance at $p < 0.05$ (two-tailed). The TCM prescription patterns for PD were analyzed according to the frequency of Chinese single herbs and Chinese herbal formulae used. All statistical analyses were run in Statistic Analysis System (SAS) software, version 9.3 (SAS Institute, Cary, NC).

RESULTS

Demographic Characteristics

Among the 9,117 patients diagnosed with PD during the period 2000–2011, there were 4,060 TCM users and 5,057 non-TCM users; of the former, 434 (10.7%) used TCM, 3,578 (88.1%) used drugs for PD, and 375 (9.2%) used TCM in combination with drugs for the treatment of PD. Sex, age, occupational status, and residential areas are factors that may affect the use of TCM. Females were more likely to use TCM than males (51.1% vs. 48.9%; adjusted OR = 1.15). The mean age of TCM users (67.2 years) was lower than that of non-TCM users (72.8 years). Most of the TCM users were older than 60 years (77.1%). Younger patients were more likely to pursue TCM therapy than older patients. The adjusted ORs were 2.61 in the 20- to 29-year-old age group, 1.38 in the 30- to 39-year-old age group, 1.82 in the 40- to 49-year-old age group, and 1.63 in the 50- to 59-year-old age group. Overall, TCM users were more likely to be female, younger, of white-collar status, and living in Central Taiwan. The adjusted ORs and 95% CIs are shown in **Table 1**.

TABLE 1 | Demographic characteristics and results of multiple logistic regression models showing the adjusted odds ratio and 95% confidence intervals for use of traditional Chinese medicine among patients with Parkinson's disease during 2000–2011 in Taiwan.

Characteristics	Non-TCM user		TCM user		*p*-value	OR (95% CI)	
	N	%	N	%		Crude	Adjusted[a]
No. of cases	5,057		4,060				
Drugs for Parkinson's disease	4,265	84.3	3,578	88.1			
TCM for Parkinson's disease			434	10.7			
TCM + drugs for Parkinson's disease			375	9.2			
Sex					<0.001		
Women	2,339	46.3	2076	51.1		1.22 (1.12–1.32)***	1.15 (1.06–1.26)**
Men	2,718	53.7	1984	48.9		1.00	1.00
Age at diagnosis of Parkinson's disease, years					<0.001		
20–29	57	1.13	118	2.91		2.89 (2.10–3.98)***	2.61 (1.87–3.65)***
30–39	137	2.71	152	3.74		1.55 (1.23–1.96)***	1.38 (1.07–1.77)*
40–49	173	3.42	254	6.26		2.05 (1.68–2.50)***	1.82 (1.48–2.24)***
50–59	316	6.25	406	10.0		1.80 (1.54–2.09)***	1.63 (1.39–1.91)***
≥60	4,374	86.5	3,130	77.1		1.00	1.00
Mean (SD)	72.8	(13.1)	67.2	(14.3)	<0.001		
Occupational status					<0.001		
White collar	1,523	30.1	1,397	34.4		1.00	1.00
Blue collar	2,137	42.3	1,684	41.5		0.86 (0.78–0.95)**	0.90 (0.81–0.99)*
Others	1,397	27.6	979	24.1		0.76 (0.69–0.85)***	0.85 (0.76–0.95)**
Residential area					<0.001		
Northern Taiwan	1895	37.5	1,511	37.2		1.00	1.00
Central Taiwan	937	18.5	954	23.5		1.28 (1.14–1.43)***	1.40 (1.24–1.57)***
Southern Taiwan	1895	37.5	1,362	33.6		0.90 (0.82–0.99)*	0.93 (0.84–1.03)
Eastern Taiwan and offshore islands	330	6.53	233	5.74		0.89 (0.74–1.06)	0.94 (0.78–1.13)

TCM, traditional Chinese medicine; SD, standard deviation; OR, odds ratio; CI, confidence interval.
[a]*Model adjusted for sex, age (categorical), occupational status, and residential area.*
*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.*

TABLE 2 | The top-10 formula Chinese herbal products (CHPs) prescribed by traditional Chinese medicine physicians for treating patients with Parkinson's disease during 2000–2011 in Taiwan (total number of CHPs, *n* = 14,794).

Formula CHPs	Number	Frequency (%)
Chaihu-Jia-Longgu-Muli-Tang	400	2.70
Ma-Zi-Ren-Wan	313	2.12
Shao-Yao-Gan-Cao-Tang	234	1.58
Tian-Wang-Bu-Xin-Dan	232	1.57
Ping-Wei-San	188	1.27
Bu-Yang-Hwan-Wu-Tang	185	1.25
Tian-Ma-Gou-Teng-Yin	176	1.19
Xue-Fu-Zhu-Yu-Tang	148	1.00
Yi-Gan-San	138	0.93
Tiao-Wei-Cheng-Qi-Tang	136	0.92

CHPs, Chinese herbal products.

Chinese Herbal Products for Parkinson's Disease

An average of 5.65 CHPs were used in a single prescription for PD patients. Most prescriptions for PD involved 4 CHPs (22.4%) each, followed by prescriptions with 5 CHPs (16.2%) and prescriptions with 3 CHPs (12.1%). The most commonly used CHPs for PD by TCM doctors are shown in **Table 2**. Chaihu-Jia-Longgu-Muli-Tang (CJLMT, 2.7%) was the most commonly used formula, followed by Ma-Zi-Ren-Wan (MZRW, 2.12%), Shao-Yao-Gan-Cao-Tang (SYGCT, 1.58%), Tian-Wang-Bu-Xin-Dan (TWBXD, 1.57%), and Ping-Wei-San (PWS, 1.27%). The most commonly used single herbs were *Uncaria tomentosa* (Willd. ex Schult.) DC. (2.84%) followed by *Gastrodia elata* Blume (2.32%), *Radix et Rhizoma Rhei* (*Rheum palmatum* L., *Rheum tanguticum* Maxim. ex Balf., *and Rheum officinale* Baill.) (1.99%), *Salvia miltiorrhiza* Bunge (1.74%), and *Polygala tenuifolia* Willd. (1.55%) (**Table 3**).

The top-three most commonly used formula CHPs of two combinations for PD were CJLMT plus MZRW (0.77%), TWBXD plus CJLMT (0.69%), and PWS plus Tiao-Wei-Cheng-Qi-Tang (0.62%) (**Table 4**). The top-three most commonly used single CHPs of two combinations were *U. tomentosa* (Willd. ex Schult.) DC. plus *G. elata* Blume (0.83%), *Acorus gramineus* Aiton plus *P. tenuifolia* Willd. (0.56%), and *Melia azedarach* L. plus *Fritillaria cirrhosa* D. Don (0.49%) (**Table 5**).

DISCUSSION

We used the NHIRD to conduct a population-based cohort study to identify the prescription patterns of CHPs for patients with PD on a nationwide scale in Taiwan. This study reveals that CJLMT and *U. tomentosa* (Willd. ex Schult.) DC. are the most commonly used formula and single herb CHPs, respectively, for PD in Taiwan.

We further analyzed the factors that may affect TCM use by PD patients (**Table 1**). Females were more likely to use TCM treatment than males, consistent with the findings of Hung et al.

TABLE 3 | The top-10 single Chinese herbal products (CHPs) prescribed by traditional Chinese medicine physicians for treating patients with Parkinson's disease during 2000–2011 in Taiwan (total number of CHPs, *n* = 14,794).

Single CHPs	Number	Frequency (%)
Uncaria tomentosa (Willd. ex Schult.) DC.	420	2.84
Gastrodia elata Blume	343	2.32
Radix et Rhizoma Rhei (*Rheum palmatum* L., *Rheum tanguticum* Maxim. ex Balf., *and Rheum officinale* Baill.)	295	1.99
Salvia miltiorrhiza Bunge	258	1.74
Polygala tenuifolia. Willd	229	1.55
Acorus gramineus Aiton	227	1.53
Astragalus mongholicus Bunge	161	1.09
Rehmannia glutinosa (Gaertn.) DC.	157	1.06
Callerya dielsiana (Harms ex Diels) P.K.Lôc ex Z. Wei and Pedley	142	0.96
Magnolia officinalis Rehder and E.H. Wilson	138	0.93

TCM, traditional Chinese medicine; CHPs, Chinese herbal products.

TABLE 4 | The top-5 most used formula Chinese herbal products (CHPs) of two combinations for Parkinson's disease during 2000–2011 in Taiwan (total number of CHPs, *n* = 14,794).

Two formula CHPs	Number	Frequency (%)
Chaihu-Jia-Longgu-Muli-Tang plus Ma-Zi-Ren-Wan	114	0.77
Tian-Wang-Bu-Xin-Dan plus Chaihu-Jia-Longgu-Muli-Tang	102	0.69
Ping-Wei-San plus Tiao-Wei-Cheng-Qi-Tang	92	0.62
Ping-Wei-San plus Wuo-Yao-Chun-Chi-San	68	0.46
Shao-Yao-Gan-Cao-Tang plus Gou-Teng-San	76	0.51

CHPs, Chinese herbal products.

TABLE 5 | The top-5 most used single Chinese herbal products (CHPs) of two combinations for Parkinson's disease in Taiwan during 2000–2011 (total number of CHPs, *n* = 14,794).

Two-combination single CHPs	Number	Frequency (%)
Uncaria tomentosa (Willd. ex Schult.) DC. *plus Gastrodia elata* Blume	123	0.83
Acorus gramineus Aiton *plus P. tenuifolia* Willd.	83	0.56
Melia azedarach L. *plus Fritillaria cirrhosa* D. Don	72	0.49
Radix et Rhizoma Rhei (*R. palmatum* L., *R. tanguticum* Maxim. ex Balf., and *R. officinale* Baill.) *plus Uncaria tomentosa* (Willd. ex Schult.) DC.	72	0.49
Melia azedarach L. *plus Aquilaria crassna* Pierre ex Lecomte	65	0.44

CHPs, Chinese herbal products.

in patients with ischemic stroke (Hung et al., 2015a). Furthermore, younger patients were more likely to use TCM than older individuals, concordant with the findings of Liao et al. in patients with chronic obstructive pulmonary disease (Liao et al., 2017). Thus, younger PD patients are more inclined to use TCM to treat their disease and maintain their daily activities, likely due to their aptitude in exploring their disease and its potential treatments. There has been no previous report regarding the relationship between TCM treatment and socioeconomic status; we found that blue-collar workers or those with other occupational statuses were less likely to seek TCM treatment than white-collar workers. Moreover, compared to the PD patients in Northern Taiwan, the PD patients in Central Taiwan tended to seek TCM treatments more often, concordant with the finding of Hung et al. that there are more TCM physicians and TCM clinics in this region than in other areas of Taiwan (Hung et al., 2015b). Therefore, people in Central Taiwan are more likely to seek combined treatment for various diseases, or to consider TCM first, than individuals residing in other areas in Taiwan.

In TCM theory, PD is characterized by tremors or muscle rigidity, which are caused by "Gan and Shan Yin deficiency," and could result in malnourishment of the muscle, leading to "Gan Qi stagnation," "Gan Yang excessive," and "Endogenous Gan wind," (Su et al., 2016). Based on the syndrome differentiation, the most common TCM syndrome patterns of PD are "Gan and Shan Yin deficiency," "deficiency of Qi and blood," "phlegm heat and wind stirring," "blood stasis and wind stirring," and "Qi stagnation and blood stasis" (Chen et al., 2017). CHPs are used depending on the syndrome differentiation. For example, Liu-Wei-Di-Huang-Wan is used for Gan and Shan Yin deficiency, and Tian-Ma-Gou-Teng-Yin is used for phlegm heat and wind stirring (Lu et al., 2016). A recent systematic review showed that several herbal extracts or their bioactive compounds had neuroprotective, neuroregenerative, and anti-oxidant properties that could reduce neuronal loss or neurodegeneration in PD models. These CHPs include *Cistanches salsa* Beck, *Scutellaria baicalensis* Georgi, *Curcuma longa* L., *Carthamus tinctorius* L., *Panax ginseng* C.A. Mey., and *Silybum marianum* (L.) Gaertn. (da Costa et al., 2017). However, as described above, the clinical indications of CHPs for specific diseases remain controversial. In our statistical analysis, we found that the most commonly used formula CHPs and herbs are CJLMT, MZRW, SYGCT, *U. tomentosa* (Willd. ex Schult.) DC., *G. elata* Blume, and *Radix et Rhizoma Rhei* (*R. palmatum* L., *R. tanguticum* Maxim. ex Balf., and *R. officinale* Baill.). The results of this study provide insights regarding personalized therapies and may form the basis for further clinical experiments and pharmacological research on the use of CHPs for the management of PD. The possible mechanisms underlying the effects of the CHPs, based on a review of the literature, are discussed below to provide insight into their pharmaceutical use.

Commonly Used Formula Chinese Herbal Products for Parkinson's Disease

CJLMT could harmonize Shaoyang, improve sleep quality, and alleviate depression. One of the mechanisms underlying depression includes decreased levels of monoamine neurotransmitters (norepinephrine, dopamine, and serotonin) and brain-derived neurotrophic factor in the hippocampus and prefrontal cortex. This effect can be reversed by the administration of antidepressants, such as monoamine oxidase inhibitors or selective serotonin reuptake inhibitors (Savegnago et al., 2007; Zhang et al., 2010). Saponins, which are extracted

from CJLMT, were found to have antidepressant-like effects in rats; they increased the concentration of serotonin and promoted the expression of brain-derived neurotrophic factor in both the prefrontal cortex and hippocampus (Li et al., 2012). Moreover, an increase in the number of apoptotic cells in the hippocampus and cortex was found in a rat depression model, while antidepressants had an anti-apoptotic effect to protect the cells (Lucassen et al., 2004). Research has found that both saponins and antidepressants can inhibit Bax and caspase-3 (the protein associated with apoptosis) expression in the hippocampus in mice, which implies that CJLMT had an antidepressant effect via neuroprotection (Liu et al., 2010). Another rapid antidepressant is ketamine, which downregulates the N-methyl-D-aspartic acid receptor, and then upregulates the signal pathway of mammalian targets of rapamycin and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors in the prefrontal cortex and brain-derived neurotrophic factor expression in the hippocampus. Research has shown that CJLMT can downregulate the N-methyl-D-aspartic acid receptor expression, thus resulting in a rapid antidepressant effect similar to that of ketamine (Wang et al., 2019).

The second most commonly used formula was MZRW, which is described to treat constipation in Shang Han Lun, and was effective against functional constipation in previous clinical trials (Zhong et al., 2013). Patients with PD have decreased phasic rectal contractions, weak abdominal wall contraction, paradoxical anal sphincter contraction during defecation, and defects in enteric nervous system dopaminergic neurons (Feldman et al., 2016). MZRW contains bowel-stimulating components, including aloe emodin, rhein, emodin, hesperidin, and paeoniflorin (Hu et al., 2015). Rhein and aloe emodin, extracted from *Radix et Rhizoma Rhei* (*R. palmatum* L., *R. tanguticum* Maxim. ex Balf., and *R. officinale* Baill.), were applied in a constipation rat model. The C_{max} and AUC of emodin in constipated rats were about ten times those of normal rats, while the $t_{1/2}$ was remarkably decreased. However, a significant decrease in the AUC values for aloe emodin and rhein was observed in constipated rats compared to normal rats. Significant differences in the main pharmacokinetic parameters were found in normal and constipated rats. The study suggested that rhein and aloe emodin directly affect intestinal cell membranes, whereas emodin indirectly affects bowel movement through adjustment of the nervous system (Gong et al., 2015). Hesperidin and paeoniflorin, extracted from *Citrus aurantium* L. and *Paeonia lactiflora* Pall., respectively, have also been shown to stimulate gastrointestinal movement via the H1 histamine receptor (Fang et al., 2009).

SYGCT is typically used for Gan dysfunction, muscle cramping, spasticity, or tremors, according to Chinese medical theory. *Paeonia lactiflora* Pall. has antispastic and analgesic effects, whereas *Glycyrrhiza glabra* L. has analgesic and anti-inflammatory effects (Takao et al., 2015). SYGCT may normalize intracellular and extracellular potassium balance by inhibiting the ultra-rapid delayed rectifier potassium current and reducing potassium efflux, while the sodium-potassium pump promotes potassium influx into myofibers. Consequently, excess potassium may be reduced in the external space of myofibers. Thus, SYGCT may balance intracellular and extracellular potassium levels, and the resulting reduction in potassium levels in the external space of myofibers could alleviate muscle pain (Suganami et al., 2014). One of the components of *Glycyrrhiza glabra* L., licochalcone A, has neuroprotective as well as anti-inflammatory effects and prevents the reduction of dopaminergic neurons in PD models by inhibiting microglia-mediated neuroinflammation (Huang et al., 2017). Paeoniflorin, another bioactive component of *Paeonia lactiflora* Pall., reduces the acidosis-induced accumulation of α-synuclein and promotes autophagic degradation of α-synuclein by regulating both the expression and activity of acid-sensing ion channels, which are ligand-gated cation channels that respond to acidic stimuli (Sun et al., 2011). α-Synuclein aggregation is associated with PD pathophysiology and results in intracellular oxidative stress, inflammation, and apoptosis; thus, *Paeonia lactiflora* Pall. protects against α-synuclein cytotoxicity (Shah et al., 2014).

TWBXD is commonly used for insomnia in TCM practice. A systematic review showed the promising effect of TWBXD regarding alleviating insomnia (Yang et al., 2019). PWS is a commonly used medication for gastrointestinal disorders in TCM practice. The mechanisms of Western medicines for the treatment of functional dyspepsia include acid suppression, gastric mucosa protection, gastric motility promotion, and anti-*Helicobacter pylori* activity. Both clinical and laboratory research studies indicate that liver stagnation and spleen deficiency are the main syndromes of functional dyspepsia. Substance P is one of the most important brain–gut peptides in mammals and is composed of 11 amino acids. It is mainly distributed in the central nervous system, spinal cord dorsal root, and ENS. It promotes gastrointestinal peristalsis, protects the gastrointestinal epithelium to repair the impaired gastrointestinal mucosa, and causes gastric and intestinal mechanical hypersensitivity (Lin et al., 2012). PWS had therapeutic effects in rats with functional dyspepsia by influencing both brain–gut substance P and vasoactive intestinal peptide levels (Du et al., 2018).

Commonly Used Single Chinese Herbal Products for Parkinson's Disease

U. tomentosa (Willd. ex Schult.) DC. was the most commonly used single herb for PD treatment; it relieves "Gan Yang excessive" and "endogenous Gan wind" according to Chinese medical theory. *U. tomentosa* (Willd. ex Schult.) DC. has protective effects on dopaminergic neurons by reducing reactive oxygen species (ROS) generation; it increased glutathione levels and inhibited caspase-3 activity in a PD cell model (Shim et al., 2009).

The second most commonly used single herb was *G. elata* Blume. *G. elata* Blume decreased L-3,4-dihydroxyphenylalanine (L-DOPA)-induced dyskinesia in a PD mouse model by inhibiting phosphorylated extracellular regulated protein kinases (pERK) and FBJ murine osteosarcoma viral oncogene homolog B (FosB) expression, which are abnormally activated by

TABLE 6 | Possible mechanisms underlying the effects of frequently used Chinese herbal products (CHPs) for Parkinson's disease, ranked by prevalence of use.

Formula CHPs	Components	Mechanisms or effects
Chaihu-Jia-Longgu-Muli-Tang	*Bupleurum chinense* DC., *Zingiber officinale* Roscoe, *Scutellaria baicalensis* Georgi, *Panax ginseng* C.A. Mey., *Cinnamomum cassia* (L.) J.Presl, *Pinellia ternata* (Thunb.) Makino, *Radix et Rhizoma Rhei* (*R. palmatum* L., *R. tanguticum* Maxim. ex Balf., and *R. officinale* Baill.), *Ziziphus jujuba* Mill.	CJLMT is commonly used for insomnia and depression in TCM practice. CJLMT reduced monoaminergic neurotransmitter system signaling and enhanced the expression of brain-derived neurotrophic factor in an animal study Li et al. (2012). Saponin, a component extracted from CJLMT, decreases expression of Bax and caspase-3 proteins in the hippocampus, thereby preventing neuronal apoptosis due to stress Liu et al. (2010). The antidepressant effect of CJLMT is probably due to reversal of the abnormal expression of N-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor in the prefrontal cortex according to a mouse study Wang et al. (2019).
Ma-Zi-Ren-Wan	*Cannabis sativa* L., *Citrus aurantium* L., *Magnolia officinalis* Rehder and E.H.Wilson, *Radix et Rhizoma Rhei* (*R. palmatum* L., *R. tanguticum* Maxim. ex Balf., *and R. officinale* Baill.), *Prunus armeniaca* L., and *Paeonia lactiflora* Pall.	MZRW is commonly used for constipation in TCM practice. Rhein, emodin, and aloe emodin affected intestinal cell membranes and bowel movement by modulating the bowel nervous system to relieve constipation in an animal study Gong et al. (2015). Hesperidin and paeoniflorin can stimulate gastrointestinal movement via the H1 histamine receptor Fang et al. (2009).
Shao-Yao-Gan-Cao-Tang	*Paeonia lactiflora* Pall. and *Glycyrrhiza glabra* L.	SYGCT is commonly used for pain control or rigidity in TCM practice. *Paeonia lactiflora* Pall. has antispastic and analgesic effects, whereas *Glycyrrhiza glabra* L. has analgesic and anti-inflammatory effects Takao et al. (2015). SYGCT may balance intracellular and extracellular potassium levels, resulting in reduction of potassium levels in the external space of myofibers, which can result in improvement of muscle pain Suganami et al. (2014). Licochalcone A has neuroprotective and anti-inflammatory effects and prevents the reduction of dopaminergic neurons in animal PD models by inhibiting microglia-mediated neuroinflammation Huang et al. (2017). Paeoniflorin promotes autophagic degradation of α-synuclein by regulating the expression of acid-sensing ion channels Sun et al. (2011).
Tian-Wang-Bu-Xin-Dan	*Asparagus cochinchinensis* (Lour.) Merr., *Panax ginseng* C.A. Mey., *Scrophularia ningpoensis* Hemsl., *S. miltiorrhiza* Bunge, *P. tenuifolia* Willd., *Platycodon grandiflorus* (Jacq.) A.DC., *Angelica sinensis* (Oliv.) Diels, *Schisandra chinensis* (Turcz.) Baill., *Liriope spicata* Lour., *Platycladus orientalis* (L.) Franco, and *Rehmannia glutinosa* (Gaertn.) DC.	TWBXD is commonly used for insomnia in TCM practice. A systematic review showed the promising effect of TWBXD in alleviating insomnia Yang et al. (2019).
Ping-Wei-San	*Magnolia officinalis* Rehder and E.H.Wilson, *Citrus aurantium* L., *Atractylodes lancea* (Thunb.) DC., and *Glycyrrhiza glabra* L.	PWS is a common medication for gastrointestinal disorders in TCM practice. PWS had therapeutic effects in rats with functional dyspepsia by influencing brain–gut substance P and vasoactive intestinal peptide Du et al. (2018).
Single CHPs		
Uncaria	*Uncaria tomentosa* (Willd. ex Schult.) DC.	*Uncaria tomentosa* (Willd. ex Schult.) DC. is commonly used for headache, dizziness, and muscle twitching in TCM practice. *Uncaria tomentosa* (Willd. ex Schult.) DC. had neuroprotective effects on dopaminergic neurons by inhibiting ROS generation, increasing glutathione levels, and inhibiting caspase-3 activity in a PD cell model Shim et al. (2009).
Gastrodia	*Gastrodia elata* Blume	*Gastrodia elata* Blume is commonly used for headache, dizziness, and muscle twitching in TCM practice. *Gastrodia elata* Blume decreased L-DOPA-induced abnormal involuntary movement in a PD mouse model by inhibiting pERK and FosB expressions Doo et al. (2014). *Gastrodia elata* Blume reduced apoptosis and oxidative stress by activation of the Nrf2/ARE/HO-1 signaling pathway, exerting neuroprotective effects in a cell model of PD Huang et al. (2016).
Rhubarb	*Radix et Rhizoma Rhei* (*R. palmatum* L., *R. tanguticum* Maxim. ex Balf., and *R. officinale* Baill.).	*Radix et Rhizoma Rhei* (*R. palmatum* L., *R. tanguticum* Maxim. ex Balf., and *R. officinale* Baill.) is commonly used for constipation, cellulitis, and ecchymosis in TCM practice. *Radix et Rhizoma Rhei* (*R. palmatum* L., *Rheum tanguticum* Maxim. ex Balf., and *R. officinale* Baill.) downregulated the mitogen-activated protein kinase and nuclear factor kappa B pathway, decreases nitric oxide (NO) and reactive oxygen species formation, and inhibits lipopolysaccharide-induced neuro-inflammation in an animal study Hwang et al. (2018). *Radix et Rhizoma Rhei* (*R. palmatum* L., *R. tanguticum* Maxim. ex Balf., and *R. officinale Baill.)* affected intestinal cell membranes and bowel movement by modulating the bowel nervous system to relieve constipation in a rat study Gong et al. (2015).

(Continued on following page)

TABLE 6 | (*Continued*) Possible mechanisms underlying the effects of frequently used Chinese herbal products (CHPs) for Parkinson's disease, ranked by prevalence of use.

Formula CHPs	Components	Mechanisms or effects
Salvia	*S. miltiorrhiza* Bunge	*S. miltiorrhiza* Bunge is commonly used for dysmenorrhea, irregular menstrual cycle, ecchymosis, and insomnia in TCM practice. *S. miltiorrhiza* Bunge reduces the expression of NADPH and iNOS and prevents nigrostriatal dopaminergic neurons loss Ren et al. (2015). *S. miltiorrhiza* Bunge inhibited **α**-synuclein aggregation both *in vitro* and in a PD model Ji et al. (2016).
P. tenuifolia	*P. tenuifolia* Willd.	*P. tenuifolia* Willd. is commonly used for insomnia and palpitation in TCM practice. *P. tenuifolia* Willd. inhibited ROS and NO production and thereby protected dopaminergic neurons in the substantia nigra and striatum against toxicity in a mouse study Choi et al. (2011).

CHP, Chinese herbal product.

long-term use of L-DOPA (Doo et al., 2014). *G. elata* Blume ameliorated the rotation behavior in PD rats and enhanced the expression of tyrosine hydroxylase positive neurons in the midbrain ventral tegmental area, displaying a neuroprotective effect on tyrosine hydroxylase positive neurons (Wang et al., 2013a). Another study in a cell model of PD revealed that a compound isolated from *G. elata* Blume had neuroprotective effects by reducing apoptosis and oxidative stress via activation of the nuclear factor erythroid 2-related factor 2 (Nrf2)/anti-oxidant response element (ARE)/heme oxygenase-1 (HO-1) signaling pathway (Huang et al., 2016).

Radix et Rhizoma Rhei (*R. palmatum* L., *R. tanguticum* Maxim. ex Balf., and *R. officinale* Baill.), which is the third most commonly used single herb for PD, is used for constipation, jaundice, ulcers, and hemorrhages in TCM. It has been shown to exert anti-neuro-inflammatory effects by downregulating the mitogen-activated protein kinase and nuclear factor kappa B pathway, decreasing nitric oxide (NO) and ROS formation and inhibiting lipopolysaccharide-induced neuroinflammation in an animal study (Hwang et al., 2018). Rhein and aloe emodin are components with effects on bowel movements, as stated above.

Salvia miltiorrhiza Bunge is commonly used for dysmenorrhea, irregular menstrual cycles, ecchymosis, and insomnia in TCM practice. *S. miltiorrhiza* Bunge was found to inhibit α-synuclein aggregation both *in vitro* and in a PD model (Ji et al., 2016). Moreover, one of the effective components, tanshinone IIA, exerts neuroprotective effects by reducing the expression of lactaldehyde reductase (NADPH) oxidase and inducible nitric oxide synthase, which could further prevent nigrostriatal dopaminergic neuron loss (Ren et al., 2015).

P. tenuifolia Willd. is commonly used for insomnia and palpitation in TCM practice. *P. tenuifolia* Willd. exhibits anti-oxidant and anti-apoptotic activity by inhibiting ROS and NO production, which protected dopaminergic neurons in the substantia nigra and striatum against toxicity in a mouse study (Choi et al., 2011). The effects and mechanisms of the top-5 formula and single CHPs are listed in **Table 6**.

In TCM theory, "Gan Qi stagnation" is one of the TCM syndromes of PD, and it can further result in depression, anxiety, or insomnia. The frequency of use of a CHP or CHP combination represents the priority of the frequency of symptoms (Ling, 2013). CJLMT can not only relieve "Gan Qi stagnation" but also improve psychiatric symptoms and constipation (Liang and Yang, 2018). PD involves both motor and non-motor symptoms (NMSs). NMSs include mood disorders, such as apathy, anhedonia, depression, cognitive dysfunction, and hallucinosis, as well as complex behavioral disorders (Lee and Andrew, 2016; Shi et al., 2017). CJLMT is used based on the syndrome differentiation of PD, and it also relieves the NMSs of PD. Hence, it is the most commonly used CHPs formula for PD. *U. tomentosa* (Willd. ex Schult.) DC. is used for "wind stirring" which results from "Gan Qi stagnation" and "Gan and Shan Yin deficiency." *U. tomentosa* (Willd. ex Schult.) DC. is one of the major components of Tian-Ma-Gou-Teng-Yin and is used for "Gan and Shan Yin deficiency" with "wind stirring," which improves tremors and insomnia in PD patients (Yang et al., 2017). Another study showed that the combined use of CJLMT and Zhichan decoction (the latter contains *U. tomentosa* (Willd. ex Schult.) DC.) can relieve "Gan Qi stagnation" and "Gan and Shan Yin deficiency." This formula also improved insomnia, psychiatric symptoms, and tremors in PD patients (Jiang, 2019). Current research shows that *U. tomentosa* (Willd. ex Schult.) DC. has neuroprotective effects on dopaminergic neurons by inhibiting ROS generation, increasing glutathione levels; inhibiting caspase-3 activity (Shim et al., 2009); increasing cell viability and mitochondrial membrane potential; decreasing lipid peroxidation, intracellular ROS, and nitric oxide; and reducing the aggregation of α-synuclein (Shi et al., 2013). Hence, it is reasonable that *U. tomentosa* (Willd. ex Schult.) DC. is the most commonly used single CHP for PD. Our data showed that these are common PD prescriptions in Taiwan, and they may therefore be candidates for further investigations as treatments for NMSs in patients with PD.

Current research is focused on ways to increase the level of levodopa in the brain while inhibiting peripheral levodopa metabolism in order to minimize the side-effects of the medications for PD (Setty and Sigal, 2005). A meta-analysis has shown that combining TCM with dopamine-replacement therapy is generally safe and could improve the Unified Parkinson's Disease Rating Scale score in PD patients (Zhang et al., 2015). Based on our findings of the top-5 formula and single

CHPs for PD, **Table 6** shows the possible mechanisms underlying the effects of frequently used CHPs for PD, ranked by prevalence of use. These CHPs may be taken into consideration as adjunctive treatment for NMSs in patients with PD and improving the side-effects of medications for PD, according to syndrome differentiation.

A few crude medicinal herbs, over-the-counter herbal treatments, health foods containing herbs, and herbs obtained without a physician's prescription were not included in the classification surveyed in this study. Some Chinese herbal medications can be obtained from TCM pharmacies without a prescription; therefore, the frequency of CHPs usage might have been underreported in our study. However, because the NHI system has a wide coverage of TCM prescriptions, the likelihood that patients acquired substantial quantities of herbs outside of the NHI database is very small.

The NHIRD is a large, integrative database, which includes 22.60 million people out of the 22.96 million population in Taiwan. Therefore, it contained TCM prescription patterns and clinical records that were reflective of the general Taiwanese population. However, this study had several limitations. First, the effectiveness, adverse effects, and mechanisms of these CHPs for PD were not available from this study. The NHIRD could offer prescription data; however, the chart-level records, such as laboratory data and physician notes, were not available. Second, in Taiwan, approximately 5% of TCM clinics are not contracted with the NHI program; therefore, the usage rate of TCM may have been underestimated (Chang et al., 2015). Third, patients with PD present various TCM syndromes. The NHIRD did not include data that allowed differentiation between the types of TCM syndromes in PD patients and their corresponding prescriptions. Further basic research could be conducted based on the results of this study.

CONCLUSION

The most commonly used formula and single CHPs identified in this retrospective cohort study have been shown to have potential neuroprotective effects in PD by exerting anti-inflammatory, anti-oxidative, and anti-apoptotic effects. They also have effects on "Gan Qi stagnation" and "wind stirring" according to the TCM syndrome differentiation. Moreover, CJLMT has therapeutic effects on constipation, insomnia, and depression, which are common NMSs of PD. TCM adjuvant therapy may improve clinical symptoms of PD and relieve the side-effects of PD medications. Further studies are warranted to investigate the underlying mechanisms and the effectiveness of CJLMT and *U. tomentosa* (Willd. ex Schult.) DC. and to verify their use for the treatment of PD or as adjunctive medication that can be used in combination with dopaminergic agents.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The study was approved by the Institutional Review Board of China Medical University and Hospital (CMUH104-REC2-115).

AUTHOR CONTRIBUTIONS

C-EK, Y-CH, and W-LH were responsible for the study concept and design. CH and H-JC contributed to the acquisition of data. C-HL, C-EK, Y-CH, and W-LH contributed to the data analysis and interpretation of data. C-HL, HC, C-EK, and W-LH drafted the manuscript. HC, S-FH, S-YW, S-TT, and T-CW critically reviewed the manuscript. All authors approved final version for publication.

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Protective Effect of *An-Gong-Niu-Huang Wan* Pre-treatment Against Experimental Cerebral Ischemia Injury via Regulating GSK-3β/HO-1 Pathway

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An-Gong-Niu-Huang Wan (*AGNHW*), a famous formula in traditional Chinese medicine, has been clinically used for centuries for treating cerebral diseases, but the protective effects of pre-treatment with *AGNHW* on cerebral ischemia have not yet been reported. The present study aimed to test such protective effects and elucidate the underlying mechanisms on cerebral ischemia in rats by phenotypic approaches (i.e. including the neurological functional score, cerebral infarct area, neuron apoptosis, and brain oxidative stress status) and target-based approaches (i.e. involving the GSK-3β/HO-1 pathway). AGNHW was administered orally at the doses of 386.26, 772.52, and 1545.04 mg/kg respectively for 7 days to male Sprague-Dawley rats and then cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) for 1.5 h. Pre-treatment with AGNHW significantly ameliorated ischemic damage to the brain in a dose-dependent manner, including reduction of the neurological deficit score and infarct area. AGNHW pre-treatment increased the number of Nissl$^+$ cells, NeuN$^+$ and DCX$^+$ cells, and decreased the number of Tunel$^+$ cells. Moreover, AGNHW reversed the up-regulation of ROS and MDA induced by cerebral ischemia. AGNHW pre-treatment increased the expression of p-GSK-3β(Ser9)/GSK-3β (glycogen synthase kinase-3β) ratio and heme oxygenase-1 (HO-1). These results firstly revealed that short-term pre-treatment of AGNHW could significantly protect the rats from injury caused by cerebral ischemia-reperfusion, which support further clinical studies for disease prevention. The *in vivo* protective effect of AGNWH pre-treatment could be associated with its antioxidant properties by the activation of GSK-3β-mediated HO-1 pathway.

Keywords: an-gong-niu-huang wan, protective effect, cerebral ischemia, GSK-3β/HO-1 pathway, antioxidant properties

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INTRODUCTION

Ischemic stroke remains a leading cause of mortality and disability worldwide, with a huge economic burden on the society. Ischemic stroke shows a complex pathophysiological course leading to peroxidation stress, loss of membrane potential, cell depolarization and, eventually, neuronal cell death, involving a variety of distinct molecular and cellular mechanisms of ischemic brain damage (Wan et al., 2020). Although the pharmaceutical industry has been making huge strides in recent years, the lack of effective anti-stroke drugs remains one of the most unmet needs in medicine (Abe et al., 2019). Stroke is a medical emergency but medications for post-stroke treatment are extremely limited in number. A stroke usually causes lasting brain damage, long-term disability, or even death. Therefore, prevention is a more effective strategy than cure in the treatment of stroke.

Traditional Chinese medicine (TCM) with fewer side-effects is often sought to provide alternative medications to prevent ischemic stroke (Liu et al., 2018). The An-Gong-Niu-Huang Wan (AGNHW), a famous formula in TCM, composed of Arisaema Cum Bile, Rhizoma Coptidis, Bombyx Batryticatus, Radix Saposhnikoviae, Borneolum Syntheticum, Bovis Calculus, Moschus, Scorpio, Rhizoma Pinelliae Praeparatum, Radix Trichosanthis, Succinum, Radix Scutellariae, Fructus Amomi, refined honey. AGNHW has been clinically used for treating cerebral diseases for centuries. In Chinese medicine practice, AGNHW with detoxification, resuscitation, and anticonvulsant properties is clinically used for alleviating the clinical syndrome, including loss of consciousness, high fever, recurring seizures, dizziness, vomiting, movement difficulties, neck stiffness, sudden severe headaches, nausea and ataxia, aphasia, and constipation. Recent studies suggested that AGNHW exhibited a therapeutic effect on diseases of the central nervous system (Guo et al., 2014) and injury brought about by experimental cerebral ischemia (Wang et al., 2014; Tsoi et al., 2019). Nevertheless, prevention is better than cure. However, studies about the protective effect of AGNHW pre-treatment on experimental cerebral ischemia injury and its potential mechanism are still lacking.

Oxidative stress caused by reactive oxygen species (ROS) has been considered as one of the underlying mechanisms for inducing neuronal damage by ischemic stroke. Anti-oxidative stress has been considered as a promising therapeutic target and become a hot research topic on ischemic stroke prevention and treatment (Menon et al., 2020). It has been reported that potential anti-oxidant agents, such as carotenoids could be used for stroke prevention (Bahonar et al., 2017). Moreover, pretreatment with Chikusetsu Saponin IVa ameliorated cerebral ischemia reperfusion injury in diabetic mice via inhibition of GSK-3β (increase an inhibitory phosphorylation of GSK-3β at Ser9) (Duan et al., 2016). Paeonol was pre-administered intragastrically once daily for 3 days and with the last administration at 30 min before the operation in the fourth day. Pre-treatment administration of paeonol attenuated cerebral ischemic injury and reduced oxidative stress damage via upregulating expression of HO-1 in the mouse model of MCAO (Zhao et al., 2014). Therefore, the GSK-3β/HO-1 pathway was considered as the important regulator for oxidative stress. Specifically, upregulation of heme oxygenase-1 (HO-1) via GSK-3β deactivation through Ser9 phosphorylation could remarkably suppress the oxidative stress damage (Duan et al., 2019). It has been reported that glycine improved ischemic stroke via activation of the GSK-3β/HO-1 pathway (Chen et al., 2020). These studies suggested that the diminution of oxidant stress by activation of GSK-3β/HO-1 pathway is an effective therapeutic strategy for ischemic stroke prevention and treatment.

We therefore hypothesized that AGNHW pre-treatment could improve the neurological functional score, reduce the cerebral infarct area, inhibit neuronal apoptosis, attenuate the oxidative stress status, and activate the GSK-3β/HO-1 pathway in the MCAO rat model. The aim of this study was to determine whether AGNHW pre-treatment can curb stroke-associated pathological changes and neuronal death in a MCAO rat model by using phenotypic strategies (i.e. including the neurological functional score, cerebral infarct area, neuron apoptosis, brain oxidative stress status) and target-based (i.e. involving the GSK-3β/HO-1 pathway) strategies. Counteraction of oxidation via inhibition of GSK-3β may, therefore, be a promising strategy for prevention of ischemic stroke.

MATERIALS AND METHODS

Animals

The protocol of animal experiment has been officially approved by the Department of Health, the Government of the Hong Kong Special Administrative Region. The animal studies were conducted in accordance with guidelines and security standards of the Committee on the Use of Human and Animal Subjects in Teaching and Research. Male Sprague-Dawley (SD) rats weighing 200–220 g were obtained from the Beijing Viton Lihua Experimental Animal Technology Co., Ltd. (Beijing, China). The animals were kept in a humidity- and climate-controlled environment with food and water *ad libitum* and exposed to a 12–12 h light-dark cycle.

Administration of AGNHW

AGNHW was kindly provided by Ma Pak Leung Co., Ltd. According to the instruction, the dose of AGNHW used for human was 62.3 mg/kg/day. The equivalent dose for rats was 62.3 × 6.2 = 386.26 mg/kg/day which was used as the low dose of AGNHW (AGNHW-L) in the present study. The intermediate dose of AGNHW (AGNHW-M) and high-dose of AGNHW (AGNHW-H) was twice and quadruple of the clinical equivalent dose, respectively. To monitor the protective effects of AGNHW pre-treatment on experimental cerebral ischemia injury, male SD rats were randomly divided into the following five groups comprising 15 animals per group: Sham operation only, MCAO only, AGNHW-L (386.26 mg/kg) followed by MCAO, AGNHW-M (772.52 mg/kg) followed by MCAO, and AGNHW-H (1545.04 mg/kg) followed by MCAO. AGNHW was dissolved in normal saline solution and then administered intragastrically

(i.g.) once daily for 7 consecutive days. Untreated rats were treated with the equivalent volume of physiological saline.

MCAO Surgical Procedure

The MCAO surgery was performed 2 h after the last administration of AGNHW according to the previous report (Guo et al., 2014). All rats were anesthetized with 3% isoflurane via inhalation. The rats were placed in the supine position on an operating table under sterile conditions. The common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed and ligated on the left side. A monofilament suture with a silicon-coated tip (L3200, Jialing Co. Ltd., China) was inserted into the ECA and advanced through the ICA to the ostium to occlude the middle cerebral artery. Sham control rats were subjected to a similar surgical operation without occlusion. After 1.5 h of ischemia, the monofilament suture was withdrawn to permit reperfusion. The reperfusion process was continued for 24 h and then the neurological status was scored. All animals were then transcardially perfused with PBS under anesthesia to collect brain tissues for further experiments respectively: the 5 brains were used for measurements of the infarct sizes; the 5 brains were stored in 10% neutral formaldehyde solution and used for histopathological investigation; the last 5 brains were used for the measurement of reactive oxygen species (ROS), malondialdehyde (MDA) and the activation of GSK-3β/HO-1 pathway.

Functional Assessment

The neurological status of all rats ($n = 15$) was scored at 24 h after reperfusion. The degree of neurological function was assessed by the Zea-Longa score with a five-point scale: grade 0 corresponds to symptoms without neurological impairment (normal); grade 1 corresponds to inextensibility of the left forepaw when lifting the rats' tail(mild); grade 2 corresponds to circling to the left side while walking (moderate); grade 3 corresponds to walking difficulty and leaning to the left (severe); and grade 4 corresponds to inability to walk spontaneously (very severe) (Guo et al., 2019).

Measurement of Infarct Area

Rats were sacrificed after 24 h of reperfusion under anesthesia and their brains were excised for estimation of the infarct area ($n = 5$). The brains were sectioned into 2 mm slices with a rat brain matrix (RWD Life Science, Shenzhen, China) and placed in a 2% 2,3,5-triphenyltetrazoliumchloride (TTC, Sigma-Aldrich, MO, United States) solution for 20 min in the dark. Infarct size was quantified by measuring the white infarcted area and red-purple non-infarcted area using the ImageJ software. The ratio of the infarct area to the total area was calculated.

Histologic Procedures and Nissl Staining

Nissl staining was performed as previously reported (Lin et al., 2021). Twenty four hours after reperfusion, the rats ($n = 5$) were anesthetized and perfused transcardially with cold saline and 4% paraformaldehyde (PFA, Sigma-Aldrich, United States). The brains were rapidly removed, immersed in the fixative for 48 h and then embedded in paraffin and micro-sectioned into coronal slices of 5 μm thickness. The coronal brain sections were stained using 0.5% cresyl violet acetate (Beyotime, Beijing, China). The severity of neural damage was monitored by counting the number of normal neurons in infarct tissues under a Pannoramic DESK scanner from 3D-HISTECH (Hungary). The cells that contained the Nissl stain in the cytoplasm, loose chromatin and prominent nucleoli were considered to be normal neurons. Stained cells in five lesioned regions of the ischemic cortex were counted randomly.

Immunofluorescent and TUNEL Staining

Immunofluorescent staining with the appropriate primary antibodies (anti-NeuN and anti-DCX) and TUNEL staining with an in-situ Cell Death Detecting kit (Roche Diagnostics GmbH, Penzberg, Germany) were performed as previously described (Peng et al., 2019). Briefly, the brain sections were incubated with primary antibodies at 4°C overnight. After washing with PBS for three times, the sections were incubated with the appropriate secondary antibodies conjugated to fluorescein isothiocyanate. DAPI (1 μg/ml) was then used to counterstain the nuclei. For TUNEL staining, the brain sections were incubated with the TUNEL reaction mixture in the chamber at room temperature, and after washing with PBS, the slices were stained with DAPI. Optical and metric analysis of the stained tissues was performed with a Pannoramic DESK scanner from 3D-HISTECH (Hungary). All of the stained cells in the image were counted, including $NeuN^+$, $Sox2^+$ and $TUNEL^+$ cells. Stained cells in five lesioned regions of the ischemic cortex were counted randomly.

Measurements of ROS and MDA

Twenty four hours after reperfusion, infarct tissues from the rats ($n = 5$) were harvested and homogenized before use for measurements of ROS and MDA and for the Western blotting assay. The homogenate was centrifuged at 2,000 × g at 4°C for 15 min. The supernatant was evaluated to determine the content of ROS and MDA using assay kits (ROS, cat. no. E004-1-1; MDA, A003-1-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol and monitored by a spectrophotometer (UV-2600; Shimadzu Corporation, Kyoto, Japan).

Western Blotting

Proteins were then extracted from the homogenate on ice using a protein extraction reagent supplemented with a protease inhibitor (Novagen, Madison, WI, United States). Equal amounts of protein samples were separated in 10% SDS-polyacrylamide gel electrophoresis and then transferred to a PVDF membrane (Bio-Rad Laboratories). After blocking in 5% defatted milk for 1 h, the membranes were subsequently incubated with appropriate primary antibodies at 4°C overnight: p-GSK-3β(Ser9), GSK-3β, and HO-1. The membranes were then incubated with the appropriate secondary antibodies for 1 h. The β-actin protein was used as internal loading control. Images were captured using a ChemiDoc Touch imaging system (Bio-Rad Laboratories) and intensities of the protein bands were analyzed using ImageJ (NIH, United States).

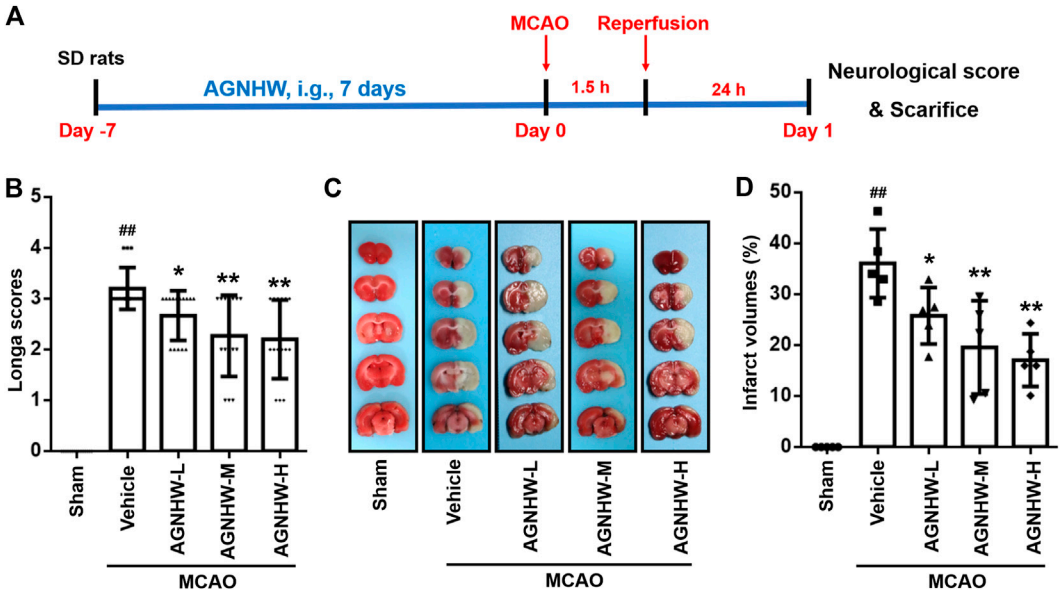

FIGURE 1 | AGNHW pre-treatment ameliorated neurological deficit and decreased infarct size in MCAO rat model **(A)** Illustration of experimental schedule. **(B)** The degree of neurological function was assessed by Zea long's scoring on a five-point scale 24 h after reperfusion ($n = 15$) **(C,D)** Infarct size was measured by TTC staining 24 h after reperfusion ($n = 5$). Representative image **(C)** and statistical analysis of results of TTC staining **(D)**. Data are means ± S.D. $^{\#\#}p < 0.01$, compared with the sham group; $^{*}p < 0.05$ and $^{**}p < 0.01$, compared with the vehicle group.

Statistical Analyses

The quantitative results are presented as the means ± standard deviation (SD). Data were analyzed statistically by using one-way analysis of variance, and difference between groups was considered statistically significant at $p < 0.05$ or smaller. Image analysis and cell counting were performed in ImageJ. All graphs were produced using the GraphPad Prism 5.0 software (San Diego, CA, United States).

RESULTS

Protective Effect of Pre-treatment With AGNHW on Injury Induced by Experimental Cerebral Ischemia

The protective effect of AGNHW pre-treatment was monitored by amelioration of neurological deficits and reduction of infarct area. Neurological deficit was evaluated by Zea long's scoring at 24 h after reperfusion. The neurological scores were significantly higher in the MCAO group than the sham group. Compared with the MCAO group, the neurological scores were markedly reduced in the AGNHW groups in a dose-dependent manner. Statistically significant difference was observed 24 h after reperfusion between the sham group and groups treated with the various doses of AGNHW (386.26, 772.52 and 1545.04 mg/kg) (**Figures 1A,B**). Additionally, the infarct areas in the MCAO rats were measured by TTC staining. As shown in **Figures 1C,D** no infarct was observed in the sham group, while rats in the MCAO group showed extensive lesions in both striatum and lateral cortex 24 h after reperfusion. Compared with the MCAO group, the infarct areas in the MCAO rats pre-treated with the various doses of AGNHW (386.26, 772.52 and 1545.04 mg/kg) were reduced significantly in a dose-dependent manner.

AGNHW Pre-treatment Mediated Neuroprotection

Nissl staining and immunofluorescent staining were performed to visualize the expression of NeuN and DCX in ischemic penumbra of cortex after 24 h of reperfusion. Regarding Nissl staining, the percentage of Nissl-positive cells in the ischemic cortex in the MCAO group was significantly decreased compared with the Sham group. After pre-treatment with AGNHW, the percentage of Nissl-positive cells was significantly increased in a dose-dependent manner (**Figures 2A,B**). Additionally, most of the Nissl-positive cells in MCAO group underwent a reduction in size with an increased intercellular space, which was ameliorated after AGNHW pre-treatment (**Figure 2A**). The same tendency was also observed in immunofluorescent staining. Specifically, the percentages of NeuN-positive and DCX-positive cells were significantly reduced in the MCAO group relative to the Sham group, which was significantly ameliorated after AGNHW pre-treatment in dose-dependent manner (**Figures 2C,D**). These results indicated the pre-treatment with AGNHW significantly restored the neural loss in injury associated with experimental cerebral ischemia.

AGNHW Pre-treatment Inhibited Cellular Apoptosis

Cellular apoptosis in the ischemic cortex was analyzed by TUNEL staining 24 h after reperfusion. There was a marked increase in

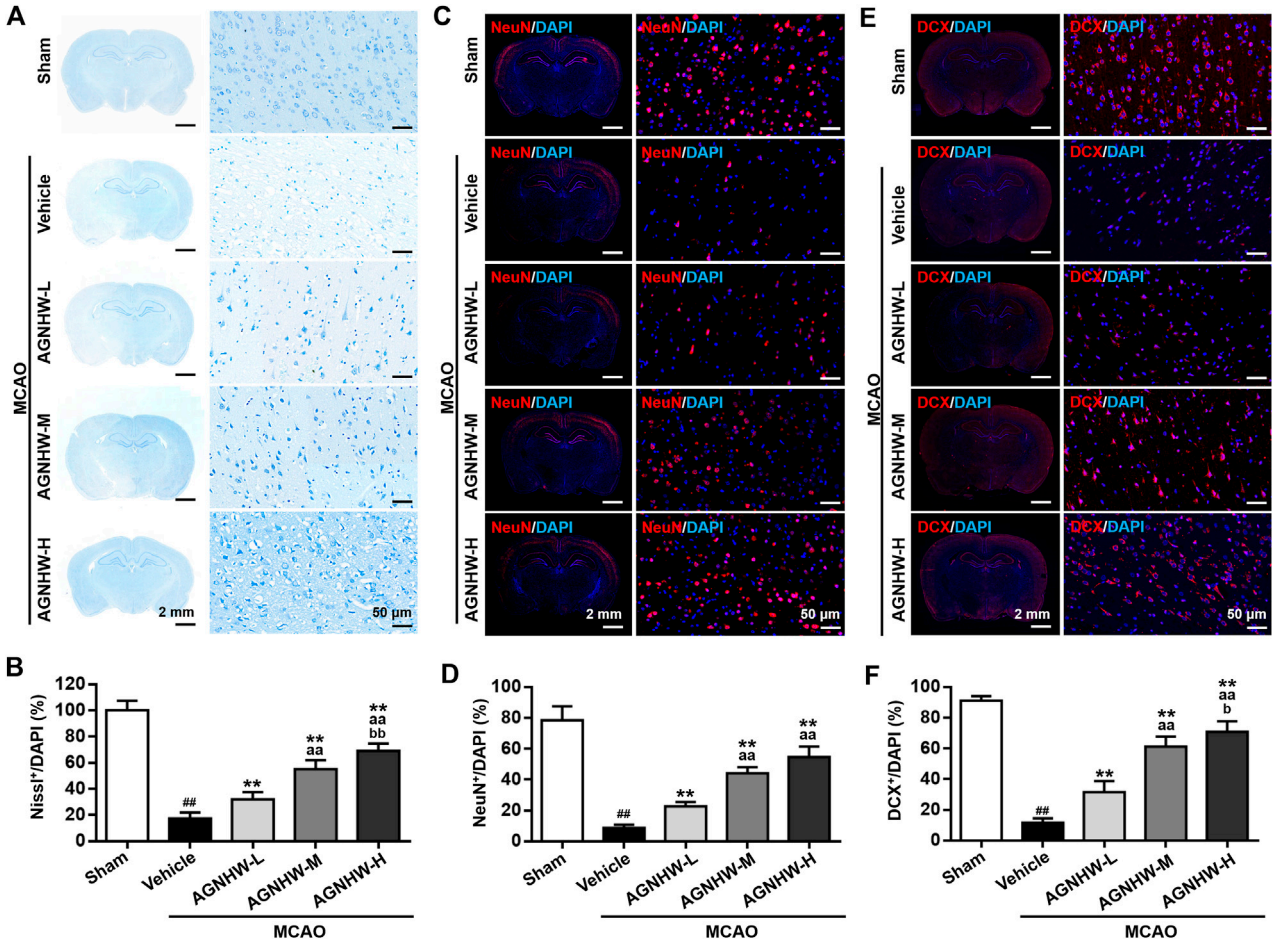

FIGURE 2 | AGNHW pre-treatment restored neuronal loss in MCAO model **(A,B)** Representative images (**A**) and statistical analysis results (**B**) of Nissl staining in the infarct area of the ischemic cortex in MCAO rats with or without pre-treatment with AGNHW **(C,D)** Representative images (**C**) and statistical analysis results (**D**) of immunofluorescent staining to visualize NeuN in the infarct area of the ischemic cortex in MCAO rats with or without pre-treatment with AGNHW **(E,F)** Representative images (**E**) and statistical analysis results (**F**) of immunofluorescent staining to visualize DCX in the infarct area of the ischemic cortex in MCAO rats with or without pre-treatment with AGNHW. $N = 5$ rats per group. Data are means ± S.D. $^{\#\#}p < 0.01$, compared with the sham group; $^{**}p < 0.01$, compared with the vehicle group; $^{aa}P<0.01$, compared with AGNHW-L group; $^{b}P<0.05$ and $^{bb}P<0.01$, compared with the AGNHW-M group.

the number of TUNEL-positive cells in the ischemic cortex in the MCAO group compared with the sham group. In contrast, the groups treated with AGNHW demonstrated a significant dose-dependent reduction TUNEL staining compared with the MCAO group. Specifically, the percentage of TUNEL-positive cells was significantly reduced in the AGNHW-H group compared with the AGNHW-M group which was in turn significantly lower than that of the AGNHW-L group (**Figures 3A,B**). These results strongly suggested that cell apoptosis was attenuated by AGNHW pre-treatment.

AGNHW Pre-treatment Mitigated Oxidative Stress–Elicited Damage

To investigate the effect of pre-treatment with AGNHW on the damage brought about by oxidative stress in MCAO rats, the levels of ROS and MDA were determined. The level of MDA is an index of lipid peroxidation as the biomarker for oxidative stress. Both ROS and MDA levels were significantly higher in infarct tissues of rats in the MCAO group than those in the sham group (**Figures 3C,D**). AGNHW pre-treatment exerted a significant dose-dependent effect on reducing the ROS and MDA levels in infarct tissue, indicating a protective effect of AGNHW on oxidative stress–elicited damage.

AGNHW Pre-treatment Enhanced the Activation of GSK-3β/HO-1 Pathway

The GSK-3β/HO-1 pathway was monitored by Western blot assay. As shown in **Figure 1**, cerebral ischemia for 1.5 h followed by 24 h of reperfusion downregulated the ratio of p-GSK-3β(Ser9)/GSK-3β, and markedly upregulated HO-1 expression in the rat infarct tissue. Compared with the MCAO group, AGNHW pre-treatment significantly up-regulated the expression ratio of p-GSK-3β(Ser9)/GSK-3β, indicating the inactivation of GSK-3β, and the expression level of HO-1 at

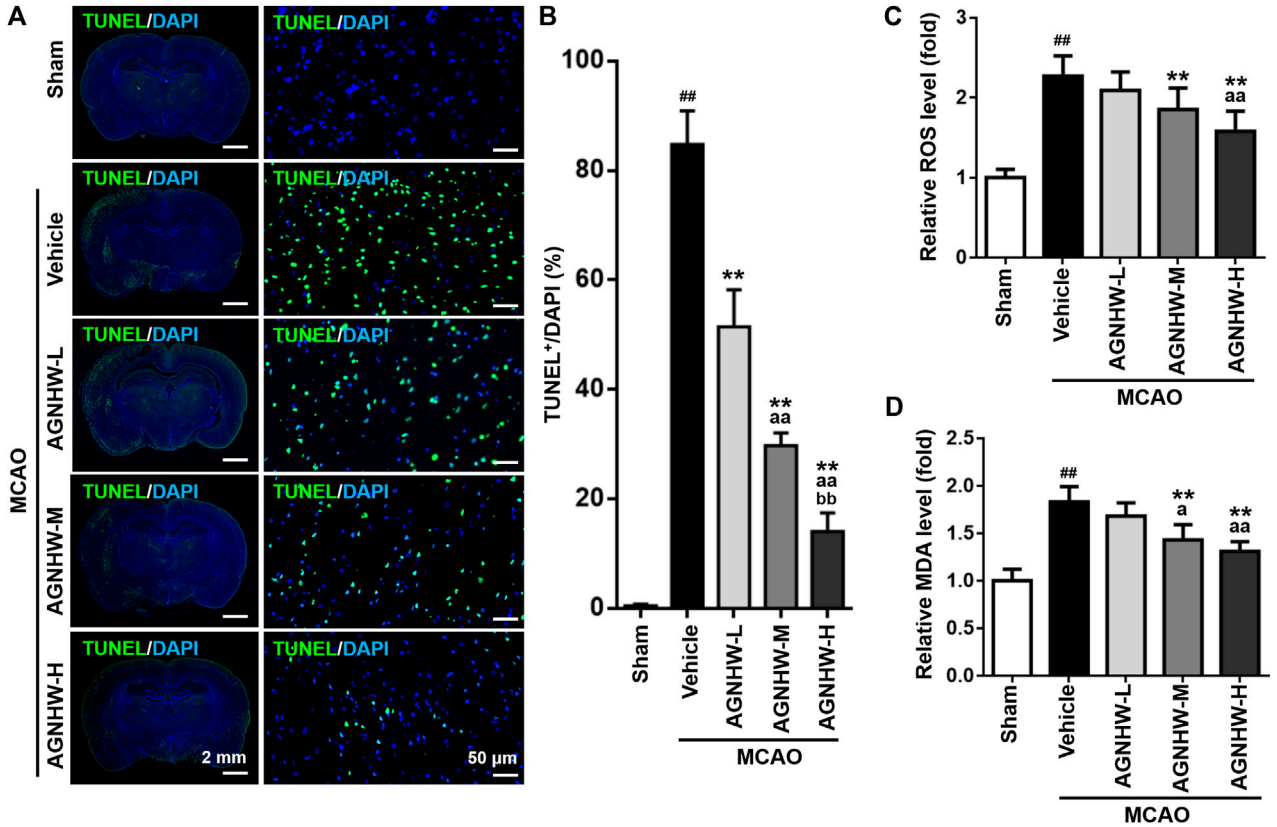

FIGURE 3 | Pre-treatment with AGNHW suppressed cell apoptosis and oxidative stress damage in MCAO model **(A,B)** Representative images (**A**) and results of statistical analysis (**B**) of TUNEL staining in the infarct area of the ischemic cortex in MCAO rats with or without pre-treatment with AGNHW **(C, D)** The status of oxidative stress was monitored by cerebral ROS (**C**) and MDA (**D**) levels, analyzed by appropriate assay kits. *N* = 5 rats per group. Data are means ± S.D. $^{\#\#}p < 0.01$, compared with sham group; $^{**}p < 0.01$, compared with vehicle group; $^{a}P < 0.05$ and $^{aa}P<0.01$, compared with AGNHW-L group; $^{bb}P<0.01$, compared with AGNHW-M group.

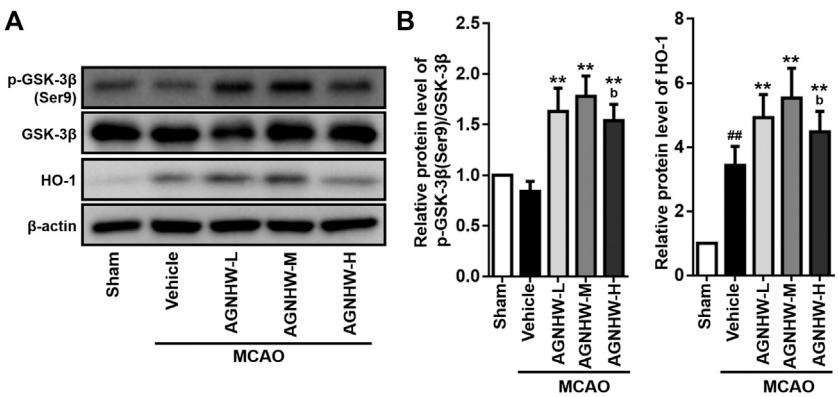

FIGURE 4 | Pre-treatment with AGNHW enhanced activation of GSK-3β/HO-1 pathway. Western blot analysis **(A)** and the normalized protein levels **(B)** of the indicated protein markers of the GSK-3β/HO-1 pathway in the infarct area of the ischemic cortex in MCAO rats with or without AGNHW pre-treatment. AGNHW pre-treatment significantly increased the ratio of *p*-GSK-3β(Ser9)/GSK-3β and the expression of HO-1 in the infarct area of MACAO rats. Full length blots are shown in **Supplementary Figure S1**. *N* = 4 rats per group. Data are means ± S.D. $^{\#\#}p < 0.01$, compared with sham group; $^{**}p < 0.01$, compared with vehicle group; $^{b}P<0.05$, compared with AGNHW-M group.

24 h after reperfusion with the highest peak appeared in the AGNHW-M (772.52 mg/kg) group (**Figure 4**). These results indicated that AGNHW pre-treatment significantly activated the GSK-3β/HO-1 pathway in MCAO rats.

DISCUSSION

Stroke is the third leading cause of death in China ranking just after malignant tumours and heart disease and the top 10 causes of death worldwide (Wang et al., 2020). To date, the vast majority of pharmacological treatments fails to effectively ameliorate the sequelae of ischemic stroke. A promising strategy underway utilizes tissue plasminogen activator (tPA) as a thrombolytic therapeutic. However, significant side effects limit its use to only a small number of patients. Furthermore, the therapeutic time window of tPA is extremely limited and requires more specialized equipment for successful implementation (Tseng et al., 2020). Consequently, the bulk of stroke patients does not receive any specific pharmacological therapy, ensuing in high mortality and disability rates. Hence, it is imperative to devise potential neuroprotective or therapeutic strategies to forestall or treat ischemic stroke.

Prevention is a better strategy than cure in the treatment of stroke. In this study, we firstly demonstrated that pre-treatment with AGNHW for 7 days prior to MCAO significantly improved the neurological functional score and reduced the cerebral infarct area in the MCAO rat model in a dose-dependent manner. Additionally, in the present study, the human equivalent dose of AGNHW (low-dose group, 386.26 mg/kg/day) displayed a pronounced protective effect on the rat model of MCAO. Mechanistic studies proved that pre-treatment with AGNHW could increase an inhibitory phosphorylation of GSK-3β at Ser 9 without affecting the expression level of total GSK-3β protein and subsequently promote the protein expression of its downstream target HO-1. A number of studies have indicated that inhibition of GSK-3β ameliorated cerebral ischemia injury (Chuang et al., 2011; Venna et al., 2015; Pang et al., 2016; Wang et al., 2017; Gao et al., 2020; Wen et al., 2020), consistent with our findings. Therefore, the effect of AGNHW pre-treatment might be attributed to its protective action on neurons against oxidative stress damage via activation of the GSK-3β-mediated HO-1 pathway.

The antioxidant response element (ARE)–mediated antioxidant pathway plays an important role in maintaining the redox status. Heme oxygenase-1 (HO-1) has been reported to have the most AREs in its promoter, making it a promising therapeutic target against brain injury in cerebral infarction (Bereczki et al., 2018). GSK-3β has emerged as the integration point and is pivotal in switching off the self-protective antioxidant stress response, thus dictating the magnitude and duration of the HO-1 antioxidant response. It has been proven in many studies that inhibition of GSK-3β effectively upregulates HO-1 protein expression (Jiang et al., 2015; Yan et al., 2020). As a result, enhancement of HO-1 via GSK-3β inactivation (GSK-3β/HO-1 pathway) plays a crucial role in improving ischemic stroke (Chen et al., 2020). In summary, HO-1 up-regulation is an adaptive response to oxidative stress in the body. The relationship between GSK-3β and HO-1 pathway in the process of cerebral ischemia is shown as follows: significant amounts of oxidants are generated during cerebral ischemia/reperfusion, and oxidative stress causes brain damage after stroke.

In this study, we found that the expression level of HO-1 was dramatically increased by 1.5 h of cerebral ischemia followed by 24 h of reperfusion, consistent with the previous report that increased HO-1 activity begin immediately after ischemia and continue for 24 h (Shah et al., 2019). Further overexpression of HO-1 by AGNHW pre-treatment reduced oxidative stress in a rat model of ischemic stroke. Therefore, inactivation of GSK-3β could serve as a novel therapeutic target for the protection of stroke and this needs further studies.

Some studies revealed that the inhibition of GSK3β phosphorylation at Tyr 216 deactivated GSK-3β, resulting in a benefit for cerebral ischemia (Chen et al., 2016; Wang et al., 2019). On the other hand, the regulation of GSK-3β usually also depends on phosphorylation within the amino-terminal domain of GSK-3β (Ser 9), resulting in inactivation of GSK-3β. In our present study, we demonstrated that pre-treatment with AGNHW significantly inhibited GSK-3β activation by enhancing GSK-3β phosphorylation at Ser9. A number of studies have indicated that inhibition of GSK-3β contributed to an amelioration of cerebral ischemia injury (Chuang et al., 2011; Venna et al., 2015; Pang et al., 2016; Wang et al., 2017; Gao et al., 2020; Wen et al., 2020), which is consistent with our findings. In a further study, we will test another hypothesis whether AGNHW pre-treatment significantly inhibits GSK-3β phosphorylation at Tyr 216 for cerebral ischemia, before performing the clinical study. Some studies have shown that inhibition of GSK3β phosphorylation is beneficial to cerebral ischemia, the differences between this and the paragraph above are shown as follows: the regulation of GSK-3β usually depends on phosphorylation at its Ser 9 and/or Tyr 216.

It was reported that *Rhizoma Coptidis,* an important traditional Chinese herb in AGHNW, was used for aging-related diseases treatment via antioxidative effect by HO-1 activation (Xu et al., 2017). It was also found that *baicalin* and *baicalein* are flavonoids extracted from *Scutellaria baicalensis*, another traditional Chinese herb in AGHNW, could be effective in the treatment of cerebral ischemia via amelioration of oxidation stress damage (Liang et al., 2017). However, until now, it is not clear which ingredients in AGNHW are responsible for the AGNHW-induced protective effect on MCAO via GSK-3β-mediated HO-1 pathway activation, which need further investigation.

Besides traditional Chinese herbs, there are some heavy metal components, such as arsenic and mercury, in AGNHW, leading to a safety risk of AGNHW. This explains the prohibition of its sale in the US and European markets. In the present study, the commercially available product AGNHW without cinnabar and realgar has been used in this study due to the safety concerns. No significant toxicity was detected during the administration of AGNHW. Additionally, it was reported that the expression of biomarker of metal toxicity metallothionein-1 was not altered by AGNHW (used at 6-fold of the clinical dosage) which was orally

administered daily for six weeks in the mouse model (Lu et al., 2011). Published clinical evidence from 1974 to 2015 indicates that the risk of adverse drug reactions and adverse events from AGNHW administration was very low (Zhao et al., 2015). Although some studies also suggested that subchronic use of cinnabar or realgar-containing herbal products could elicit mild renal injury (Wang et al., 2015; Luo et al., 2017), it has been reported it is not very likely that usage of AGNHW at a regular dose for a short duration would induce the accumulation of arsenic and mercury and influence liver and kidney functions (Tsoi et al., 2019). Thus, we conclude that AGNHW is relatively safe when used in short term for ischemic stroke, and it is strongly desirable to have well-designed experiments to systematically study the toxicological effects of prolonged usage of AGNHW (with cinnabar and realgar).

In the present study, AGNHW brought about a dose-dependent decline in the number of TUNEL-positive apoptotic cells in the ischemic brain cortex. This is in keeping with the earlier demonstration that AGNHW administration protected rats from cerebral ischemia–reperfusion injury induced by MCAO. The reduction in the percentages of Nissl-positive, NeuN-positive and DCX-positive cells caused by MCAO was alleviated by AGNHW. AGNHW treatment up-regulated Bcl-2 expression (Wang et al., 2014; Tsoi et al., 2019) and down-regulated the expression of Bax (Wang et al., 2014; Tsoi et al., 2019), caspase-3 (Wang et al., 2014), $p47_{phox}$, inducible nitric oxide synthase, and 3-nitrotyrosine in the ischemic brains (Tsoi et al., 2019). In the present investigation, AGNHW pre-treatment exerted a significant dose-dependent effect on reducing the levels of reactive oxygen species and malondialdehyde in infarct tissue in the ischemic brain cortex, indicating a protective effect of AGNHW on oxidative stress–elicited damage. Tsoi et al. showed that AGNHW exerted neuroprotective effects and minimized cerebral ischemia–reperfusion injury e.g. alleviated oxidative and nitrative (peroxynitrite) stress-mediated matrix metalloproteinase activation and protecting tight junction proteins ZO-1 and claudin-5, which are important components in maintaining the blood-brain barrier. (Tsoi et al., 2019). Malondialdehyde level was elevated and total antioxidant power was diminished in stroke patients compared with control subjects (Menon et al., 2020). AGNHW suppressed lipopolysaccharide-induced reduction of dopamine uptake; inhibited formation of intracellular reactive oxygen species and the mRNA expression of cyclooxygenase-2, inducible nitric oxide synthase, interleukin-1beta and tumor necrosis factor-alpha, and the release of interleukin-1beta, tumor necrosis factor-alpha, and prostaglandin E2 and the protein level of inducible nitric oxide synthase (Li et al., 2018). Data from the various research laboratories all indicated a beneficial action of AGNHW on the ischemic brain.

In conclusion, the present report is the first which demonstrates that pre-treatment of a rat model of ischemic stroke with AGNHW for 7 consecutive days effectively curtailed the injury done in the ischemic brain via activation of GSK-3β-mediated HO-1 pathway. The GSK-3β inhibitor TWS119 given intraperitoneally exhibited a mitigating effect on damage induced by MCAO (Wang et al., 2017). As a neuroprotective agent HO-1 minimizes the damage caused by cerebral ischemia (Song et al., 2019). Our findings strongly suggested that AGNHW pre-treatment has the potential to exert a protective effect *in vivo* against ischemic stroke or other relative neuronal diseases, and inactivation of GSK-3β could serve as a novel therapeutic target for the protection against stroke, which support further clinical studies for disease prevention.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal experiment protocol was officially approved by the Department of Health, Government of the Hong Kong Special Administrative Region. The animal studies were conducted in accordance with the guidelines and security standards of the Committee on the Use of Human and Animal Subjects in Teaching and Research.

AUTHOR CONTRIBUTIONS

KY, SS, and SZ designed and conceived the study. XJ, YW and KL conducted the experiments. ZZ and PZ conducted the data analysis. SZ and XJ wrote the manuscript. KY, SS, MN and SQ provided constructive comments. KY, SS and MN rewrote parts of manuscript. All authors have read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.640297/full#supplementary-material.

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REVIEW
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Resveratrol in Rodent Models of Parkinson's Disease: A Systematic Review of Experimental Studies

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Parkinson's disease (PD) is a common neurodegenerative disease featured by progressive degeneration of nigrostriatal dopaminergic neurons (DA) accompanied with motor function impairment. Accumulating evidence has demonstrated that natural compounds from herbs have potent anti-PD efficacy in PD models. Among those compounds, resveratrol, a polyphenol found in many common plants and fruits, is more effective against PD. Resveratrol has displayed a potent neuroprotective efficacy in several PD animal models. However, there is still no systematic analysis of the quality of methodological design of these studies, nor of their results. In this review, we retrieved and analyzed 18 studies describing the therapeutic effect of resveratrol on PD animal models. There are 5 main kinds of PD rodent models involved in the 18 articles, including chemical-induced (MPTP, rotenone, 6-OHDA, paraquat, and maneb) and transgenic PD models. The neuroprotective mechanisms of resveratrol were mainly concentrated on the antioxidation, anti-inflammation, ameliorating mitochondrial dysfunction, and motor function. We discussed the disadvantages of different PD animal models, and we used meta-analysis approach to evaluate the results of the selected studies and used SYRCLE's risk of bias tool to evaluate the methodological quality. Our analytical approach minimized the bias of different studies. We have also summarized the pharmacological mechanisms of resveratrol on PD models as reported by the researchers. The results of this study support the notion that resveratrol has significant neuroprotective effects on different PD models quantified using qualitative and quantitative methods. The collective information in our review can guide researchers to further plan their future experiments without any hassle regarding preclinical and clinical studies. In addition, this collective assessment of animal studies can provide a qualitative analysis of different PD animal models, either to guide further testing of these models or to avoid unnecessary duplication in their future research.

Keywords: Parkinson's disease, resveratrol, neuroprotective effects, PD animal models, meta-analysis

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Abbreviations: 6-OHDA, 6-hydroxydopamine; BBB, blood-brain barrier; CAT, catalase; DA, dopaminergic neurons; ELISA, enzyme-linked immunosorbent assay; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; HPLC, high-performance liquid chromatography; IF, immunofluorescence; IHC, immunohistochemistry; LB, Lewy bodies; MPP^+, 1-methyl-4-phenylpyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PC, protein carbonyl; PD, Parkinson disease; SD, Sprague–Dawley; SIRT1, sirtuin 1; SNpc, substantia nigra pars compacta; SOD, superoxide dismutase; SOCS-1, suppression of cytokine signaling-1; TH, tyrosine hydroxylase; WB, Western blotting.

INTRODUCTION

Parkinson's disease (PD) is a prevalent neurodegenerative disease with defective motor function. Around the globe, 2–3% of people aged over 65 suffer from PD. People with PD may have trouble in walking, talking, or doing simple tasks (Poewe et al., 2017). Disabilities of the motor system, including stiffness, bradykinesia, tremor, and unstable posture are the main syndromes of PD (Dickson, 2018; Homayoun, 2018). The major neuropathological marks of PD are neuronal loss and the accumulation of Lewy bodies (LBs). LBs consist of misfolded and aggregated α-synuclein which is involved in synaptic transportation (Wang et al., 2017). α-synuclein mainly aggregates in neurons, and current research has shown that its toxic conformations are protofibrils and oligomers (Lashuel et al., 2013). Moreover, mitochondrial deficits and neuroinflammation also appear to be related to the pathogenesis of PD (Greenamyre et al., 1999; Hirsch and Hunot, 2009).

Currently, strategies for treating PD can be classified into five types as follows: gene therapy, neuroprotective drugs, anti-inflammatory drugs, stem cells, and neurotrophic factors. Some of the methods (e.g., stem cells) are prohibitively expensive for normal people; and all have side effects to some degree. Therefore, there is a need of safe, effective, and affordable drugs that can be administered over the long term. Resveratrol (3,4,5-trihydroxy-*trans*-stilbene) is a natural polyphenol which was first isolated from the roots of *Veratrum grandiflorum* Loes (white hellebore). It was also extracted from the roots of *Polygonum cuspidatum* which was commonly used in traditional Chinese and Japanese medicine (Baur and Sinclair, 2006). Currently, resveratrol can be found in a variety of plants such as *P. quinquefolia* (L.) Planch, *Paeonia lactiflora*, and *Morus alba* (Chun-Fu et al., 2013), and in several common foods like berries, peanuts, red wine, and red grapes (Oliveira et al., 2017). A variety of experiments have indicated that resveratrol appears to have ameliorating effects on PD models (Okawara et al., 2007), and many types of research have been operated to look into these effects. However, many of the studies have methodological flaws that make their results and conclusions questionable. For example, some studies showed bias in their research design. Other studies are hard to compare because of methodological differences; for instance, several studies used the same toxin to induce the PD animal models, whereas with different administration times and dosages. Besides, different researchers proposed different mechanisms as to the neuroprotection of resveratrol (Guo et al., 2016; Huang et al., 2019). However, a sole research cannot elucidate all the details of the mechanisms of resveratrol on PD. Addressing all these problems, this review systematically analyzes the bias of each study, and then summarizes the mechanisms of resveratrol, and discusses the disadvantages of different PD animal models. And this review can provide a general description to different animal models of PD, either to guide further testing of the model or to avoid unnecessary duplication. We systematically analyzed these studies aiming to ameliorate the quality of PD animal study and support some helpful information for clinical studies of resveratrol.

METHODS

Search Strategy

We searched the literature to find articles on the neuroprotective effects of resveratrol in animal PD models. We searched three data resources: "PubMed," "Web of Science," and "Google Scholar." Our search designations were "[resveratrol (Title/Abstract)] AND [Parkinson disease (Title/Abstract) OR Parkinson's disease (Title/Abstract)]". The reviewers (Li Jiang and Cheng-fu Su) evaluated the accuracy of search results independently by reading the titles and abstracts of the identified studies according to the inclusion criteria, and 18 articles were selected for this review.

Inclusion Criteria

(1) PD rodent models with no limitations as to species, gender, weight, or age were selected.
(2) The study included a control group with placebo and a resveratrol intervention group.
(3) The effectiveness of resveratrol on the PD rodent models was measured.

Exclusion Criteria

(1) Review articles, replicated articles, and abstracts without full text.
(2) No measurement of the effect of resveratrol on PD animal models.
(3) Resveratrol was combined with other chemicals or drugs in the experiments on PD animal models.

Quality Assessment

RevMan 5.3 (Cochrane Community, London, United Kingdom), ImageJ (Rawak Software Inc., Stuttgart, Germany), and SYRCLE's risk of bias tool (Hooijmans et al., 2014) were used to analyze the data.

Data Elicitation and Grade Evaluation

The specific data of these studies are provided below (**Table 1**). The information of **Table 1** includes the following details: 1) author and year of publication; 2) rodent model, including species, gender, age/weight, and modeling methods; 3) resveratrol treatment, including medication dosage, route of administration, and duration of treatment; 4) outcome measures; and 5) pharmacological activity/mechanism. We did the meta-analysis for some parts of the results of the studies by forest plot with RevMan 5.3 (Cochrane Community, London, United Kingdom).

RESULTS

Study Selection

We identified 164 references, 153 from Web of Science and PubMed data resources, and 11 coming out of Google Scholar. Of these, 144 articles were excluded after browsing titles and abstracts. In the 144 excluded articles, 46 studies did not test the

TABLE 1 | Characteristics of included research articles.

Author (Year)	Rodent model	Resveratrol treatment	Outcome measurement (change with resveratrol: ↑ or ↓)	Pharmacological activity/ mechanism
Blanchet et al. (2008)	Male C57BL/6 mice (3–4 weeks) injected with MPTP (i.p., 7 mg/kg or 10 mg/kg, 4 times at 2 h interval)	Dosage: 50 mg/kg/day; Ad: p.o.; administration time: 13 days; or Dosage: 50 mg/kg/day; Ad: p.o.; administration time: 20 days	High-performance liquid chromatography; HPLC (DOPAC, DA, HVA↑).Western blotting (WB), immunofluorescence (IF), and immunohistochemistry (IHC) (TH↑)	Increased the level of striatal tyrosine hydroxylase (TH)
Jin et al. (2008)	Sprague–Dawley (SD) rats (8–12 weeks) stereotaxic injected (right striatum) using 6-OHDA (5 μg)	Dosage: 10, 20 or 40 mg/kg/ day; Ad: p.o.; administration time: 70 days	Rotational behavior testing, ultra microstructure analysis; RT-PCR (COX-2, TNF-α↓), WB(COX-2↓)	Alleviated mitochondrial tumefaction, decreased TNF-α mRNA level, and the expression of COX-2.
Lu et al. (2008)	Male Balb/C mice (20–25 g) injected using MPTP for 7 days (i.p., 30 mg/kg)	Dosage: 20 mg/kg/day; Ad: i.v.; administration time: 7 days	Rotarod trial, grasp strength analysis. Measurement of extracellular free Radicals (DHBA↓); histology evaluation	Alleviated neuronal loss by free radical scavenging
Anandhan et al. (2010)	Male C57BL/6 mice (30–35g) injected with MPTP for 4 days (i.p., 30 mg/kg)	Dosage: 50 mg/kg/day; Ad: p.o.; administration time: 4 days	Behavioral tests (rotarod trial, hang trial, narrow beam walking test), biochemical estimations (DOPAC↑, HVA↑, TBARS↓, GSH↑, GPx↑, SOD↓, CAT↓)	Reversed toxicity of MPTP through improving the dopamine and its metabolites levels, increasing the levels of GSH, GPx, and enhancing behavior performance
Khan et al. (2010)	Male Wistar rats (16 weeks) stereotaxic injected (right striatum) with 10 μg 6-OHDA	Dosage: 20 mg/kg/day; Ad: i.p.; administration time: 15 days	Behavioral tests (rotarod trial, apomorphine-induced circling behavior, stepping test), biochemical analysis (TBARS↓, GSH↑, GPx↑, GR↑, CAT↑, SOD↑, na+/k + -atpase); HPLC (DOPAC↑, DA↑); IHC (TH↑, COX-2↓)	Improved antioxidant status and alleviated dopamine loss
Wang et al. (2011)	Male Wistar rats (180–210 g) stereotaxic injected (right striatum) with 15 μg of 6-OHDA	Dosage: 20 mg/kg/day; Ad: p.o.; administration time: 14 days	Behavioral trial (rotarod trial); IHC (TH↑); reactive oxygen species (ROS↓); apoptosis↓	Increased the total antioxidant, resveratrol liposome played a better protection role than resveratrol
Mudo et al. (2012)	PGC-1α transgenic mice injected with 14 mg/kg MPTP 3 times within a time period of 3 h, then injected with 7 mg/kg MPTP for the fourth time	Dosage: 20 mg/kg/day; Ad: i.p.; administration time: 15 days	IHC (TH↑, DAT↑); WB (TH↑, SOD2↑); HPLC (DOPAC↑, DA↑); IP of PGC-1a↑	Increased PGC-1α gene transcription; triggered neuroprotection via SIRT1/PGC-1a
Srivastava et al. (2012)	Swiss albino mice (20–25 g) only intraperitoneally injected using paraquat (10 mg/kg; ip) or injected with paraquat (10 mg/kg; ip) combined with maneb (30 mg/kg; ip), two times a week, for 9 weeks	Dosage: 10 mg/kg/day; Ad: i.p.; administration time: 63 days	RT-PCR (Cyp2d22↑, VMAT-2↑); HPLC (DA↑); IHC (TH↑); WB (TNF-α↓, Bax↓, p53↑, P-p53↓, IL-1β↓, etc.)	Ameliorated Cyp2d22 expression and paraquat accumulation, enhanced neuroprotective effect
Lofrumento et al. (2014)	C57BL/6 mice (weighing 22–24 g) injected with MPTP (i.p., 20 mg/kg, 4 doses over 8 h period)	Dosage: 50 mg/kg/day; Ad: p.o.; administration time: 21 days	IHC (TH↑), RT-PCR (TH↑, IL-1b↓, SOCS-1↑, CD11b↓, TNF-a↓, etc.); WB (TH↑, IL-1b↓, IL-6↓, SOCS-1↑, CD11b↓etc.)	Increased DA neurons by ameliorating inflammatory reactions
Wang et al. (2015)	C57BL/6 mice (9–10 weeks) injected with the 20 mg/kg MPTP per 8 h for 21 days (i.p.)	Dosage: 50 mg/kg/day; Ad: p.o.; administration time: 21 days	RT-PCR (miR-214↑and α-synuclein↓); WB (α-synuclein↓); IHC (α-synuclein↓)	Reversed expression of miR-214 and of SNCA in MPTP PD mice model
Gaballah et al. (2016)	Wistar albino rats (200–250 g) injected with rotenone every other day for 21 days (s.c. 1.5 mg/kg)	Dosage: 20 mg/kg/day; Ad: p.o.; administration time: 21 days	Catalepsy test, rotarod test; the enzyme-linked immunosorbent assay (ELISA) (DA↑, caspase-3↓, IL-1β↓); DNA binding activity (Nrf-2↑)	Improved rotenone-induced ER stress by reducing the gene expression of CHOP and GRP78 and restrained caspase-3 level, inhibited xanthine oxidase activity; preserved intracellular oxidation balance by motivating Nrf2 signaling pathway
Guo et al. (2016)	Male C57BL/6 mice (24–28 g) injected with MPTP for five days (i.p., 30 mg/kg)	Dosage: 100 mg/kg/day; Ad: p.o.; administration time: 33 days.	Behavioral tests (open-field trial, stride length test, pole test). HPLC (DOPAC↑, DA↑, HVA↑).WB and IF (TH↑, SIRT1↑, LC3B↑, p62↓, etc.)	Increased TH and dopamine levels, ameliorated behavioral impairments; activated SIRT1; triggered autophagy to degrade α-synuclein
Zhao et al. (2017)	Male C57BL/6 mice (20–25 g, 10 weeks old) administered with rotenone for 28 days (p.o. 30 mg/kg)	Dosage: 50 mg/kg/day; Ad: p.o.,; Administration time: 35 days	Rotarod test; IHC (TH↑); WB(TH↑); HPLC (DA↑); iron staining	Ameliorated motor coordination, improved iron levels, elicited neuroprotective effect
Zhang et al. (2018)	Male A53T SNCA mice (12 months)	Dosage: 50 mg/kg/day; Ad: p.o.; administration time:50 days.	Behavioral test (open-field, pole test, hindlimb clasping test, object recognition test, Y-maze test); IHC (α-synuclein↓, TH↑, Iba-1↓); ELISA (TNF-α↓, IL-6↓etc.); WB(α-synuclein↓).	Decreased neuroinflammation and oxidative stress, ameliorated motor function and cognitive deficiency in the A53T SNCA mouse model

(Continued on following page)

TABLE 1 | (*Continued*) Characteristics of included research articles.

Author (Year)	Rodent model	Resveratrol treatment	Outcome measurement (change with resveratrol: ↑ or ↓)	Pharmacological activity/ mechanism
Palle and Neerati. (2018)	Wistar rats (180–250 g) injected with rotenone for 35 days (s.c. 2 mg/kg)	Dosage:40 mg/kg/day; Ad: p.o.; administration time: 35 days	Behavioral tests (rearing behavior, rotarod test); tricarboxylic acid cycle enzymes (citrate synthase, aconitase, succinate dehydrogenase); oxidative parameters (MDA↓, GSH↑); histopathology	Altered behavioral function, reduced oxidative stress, and improved mitochondrial dysfunction.
Huang et al. (2019)	SD rats (7 weeks) stereotaxic injected with 8 μg 6-OHDA (2 μg/μL) in a unilateral midbrain substantia nigra.	Dosage: 15 or 30 mg/kg/day; Ad: p.o.; administration time: 36 days	Behavioral tests (rotarod trial, open-field trial, grid test), WB (Bcl-2↑, Bax↓, pro-caspase-3↑, PI3K↑, p-Akt↑, etc.), IHC (TH↑)	Activated the PI3K/Akt signaling pathway
Liu et al. (2019)	Balb/c mice (10 weeks) injected with MPTP (i.p., 15 mg/kg for 7 consecutive days)	Dosage: 10 mg/kg/day; Ad: p.o.; administration time:7 days.	Behavioral tests (open-field trial, rearing test), IHC (TH↑), WB (TH↑, Akt↑, α-synuclein↓, cleaved-caspase-3↓; Bax↓, Bcl-2↑, IL-1β↓)	Improved motor dysfunction, increased Bcl-2 and pAkt/Akt ratio, reduced Bax and caspase-3 level, promoted dopamine neuron survival
Xia et al. (2019)	Male mice (11–12 weeks old) injected with four doses of 20 mg/kg MPTP at 8 h interims (i.p.)	Dosage: 50 mg/kg/day; Ad: p.o.; administration time: 21 days	RT-PCR (SNCA↓, MALAT1↓, and miR-129↑); luciferase assay; WB(α-synuclein↓)	Inhibited MALAT1 expression, modulated the MALATl/mir-129/ SNCA signaling pathway

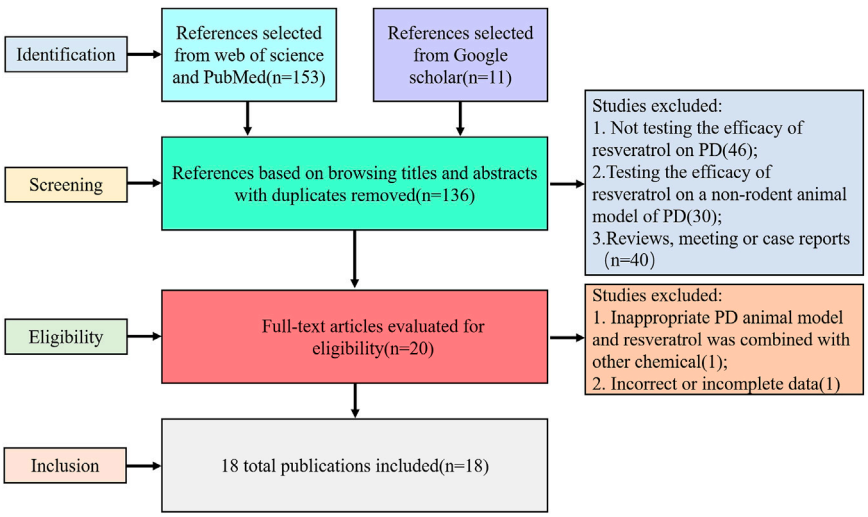

FIGURE 1 | Selection methodology for study inclusion.

efficacy of resveratrol on PD; 30 articles tested the effect on a nonrodent animal model (cell model, zebra fish, and *Drosophila*); 40 were reviews, meeting or case reports; and 28 were duplicated publications. Through reading the remaining 20 articles, we found 1 study was based on a uncommon transgenic PD model and resveratrol was combined with another chemical in the treatment, and 1 study mainly focused on the nanoparticles of resveratrol; the data were incomplete, so we excluded the 2 studies. Therefore, 18 met our criteria (the inclusion criteria and exclusion criteria described in the method) and were used for meta-analysis (**Figure 1**). This result proved the efficiency of resveratrol on PD treatment and implied us that the neuroprotective mechanism of resveratrol was still worth exploring.

Animal Models

In this review, 18 studies involved basically 2 kinds of PD rodent models: chemical-induced and transgenic animal models. Of the

TABLE 2 | Features of PD models.

Model	Mechanism	Behavior deterioration	Major usage of the model	Disadvantages
MPTP-induced	A reduction of striatal DA and TH	Dyspraxia	To generate irreversible and severe motor abnormalities	No loss of neurons from locus coeruleus; lack of age-dependent, slow progressive lesion development
Rotenone-induced	Suppresses mitochondrial complex I	Impaired motor activity	To trigger deterioration of nigrostriatal DA as well as aggregated proteins like α-synuclein	Age-independent lesions
6-OHDA-induced	Pro-oxidant properties, inhibits complex I activity	Rotational behavior	To induce motor impairment of limbs	Does not cross the BBB
Paraquat- and maneb-induced	Accelerates α-synuclein fibril formation	Impaired motor activity	To produce neuronal damage and a Parkinsonian-like syndrome	The dopaminergic toxicity is not selective
A53T transgenic	Produces mutations in SNCA	Motor deficits	Juvenile mice: usually without any overt phenotype; late middle-aged mice: development of dramatic motor phenotype	Expensive and time-consuming; the features of PD are not obvious

chemical-induced models, the 4 commonly used chemicals are as follows: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP); rotenone, 6-hydroxydopamine (6-OHDA), and paraquat (Tanner et al., 2011; Wang et al., 2020). We have listed the details of different models refereed to the 18 studies in **Table 1**. Here, we have summed up the different models in **Table 2** to better know the pathological hallmarks, and the use in practical experiments of animal PD models. The MPTP model mainly mimics three aspects of the pathogenesis of PD: deprivation of dopaminergic neurons in the SNpc, defective mitochondrial respiration, and oxidative stress (Schober, 2004). When it passes through the blood-brain barrier (BBB), it is transformed into 1-methyl-4-phenylpyridinium ion (MPP^+). This functioning metabolin is brought by dopamine shipper into DA of the SNpc where it harms the mitochondrial complex I activity, causing L-DOPA–responsive Parkinsonian syndrome, with clinical features of PD (Heikkila et al., 1984; Liberatore et al., 1999; Blum et al., 2001). However, this kind of model does not lose neurons from locus coeruleus, a classical feature of PD (Dauer and Przedborski, 2003).

Rotenone is a tropical plant extract with cytotoxic effects. It can suppress mitochondrial complex I and trigger nigrostriatal DA degeneration and aggregated proteins like α-synuclein (Sherer et al., 2003; Liu et al., 2015). The 6-OHDA–induced model is the premier PD animal model; the neurotoxic effects of 6-OHDA are mainly caused by oxidative stress which is provoked by the formulation of superoxide, hydrogen peroxide, and hydroxyl radicals, as well as it can directly inhibit the complex I mitochondrial respiratory chain (Zhuang et al., 2020). However, it does not form LB-like inclusions like those observed in PD, and it cannot cross the BBB (Jonsson, 1980; Pycock et al., 1980).

Paraquat is a quaternary nitrogen herbicide, and it produces subcellular changes associated with PD (Widdowson et al., 1996). Paraquat can accelerate α-synuclein fibril formation *in vitro*. When systematically injected into mice, paraquat can significantly increase α-synuclein levels in the brain, particularly in the frontal cortex (Widdowson et al., 1996; McCormack et al., 2002). Oxidative stress and mitochondria impairment can also be triggered by paraquat (Berry et al., 2010).

Beyond the neurotoxin models, rodent transgenic models are also very helpful in PD study. SNCA mutations lead to unusual modalities of autosome-dominant PD; indeed, SNCA was coined to be linked with familial PD (Crabtree and Zhang, 2012). There are more than 30 known mutations (including E46K, A53T, and A30P) that have been recognized in SNCA gene (Meade et al., 2019). Different SNCA transgenic mice have been developed, in A53T transgenic mice, the A53T SNCA causes the formation of toxic fibrillar SNCA neuronal inclusions which can lead to motor function impairment. At the same time, the expression of the protein in catecholaminergic neurons reduces the expression of TH (Benskey et al., 2016).

Behavioral analysis motor function plays an important role in evaluating the drug's effectiveness in PD. Most of the literatures listed here (12 of 18) investigated this function of animals. The two common methods to assess motor function are rotarod test and open-field test. The rotarod test coined by Miya and Dunham in 1957 is a tool to assess the effect of a medicine on animal behavior, especially neurological effects of a drug on rodents (Bohlen et al., 2009). It consists of a rod which is turning on a fixed axis with acceleration. For animals, the time they spend on it can reflect their motor coordination. Thus, an animal's neuromuscular coordination can be assessed with the rotarod test (Shiotsuki et al., 2010). Nine research studies (50%) used the rotarod test to evaluate the protective effect of resveratrol.

Another test of coordination is the open-field test; it is popular and mainly used for rodents (Belzung, 1999). Usually, the field is marked with a grid and square intersection. The camera system can record the movement of animals, and the animals are chiefly depending on their tactual sense. Some determinants like the food or water deprivation can affect the exploration result (Prut and Belzung, 2003). In this review, 4 research studies (22%) performed the open-field test. Those articles revealed that resveratrol demonstrated a constant effect in ameliorating the motor functions in the behavioral experiments.

Nonmotor symptoms such as dementia, dysarthria, and hallucinations are also generally known in PD and may lead to notable disability (Lim and Lang, 2010). The object recognition test (ORT) is generally used to evaluate the memory and learning in rodent animals. In this test, the mice would be given 2 identical objects to observe whether they will spend more time on the novel object (Lueptow, 2017). The Y-maze test can be utilized to evaluate the short memory in mice (Kraeuter et al., 2019). In

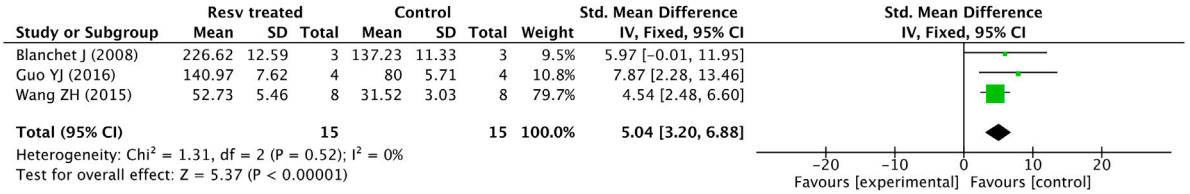

FIGURE 2 | Forest plot for TH$^+$ neuron counting measured by IHC in MPTP-induced PD mice: resveratrol vs. vehicle control. The mean difference and standard error of TH$^+$ neuron counting in the resveratrol and vehicle-treated groups were quantificationally measured by ImageJ. These data were meta-analyzed in the form of forest plot by RevMan.

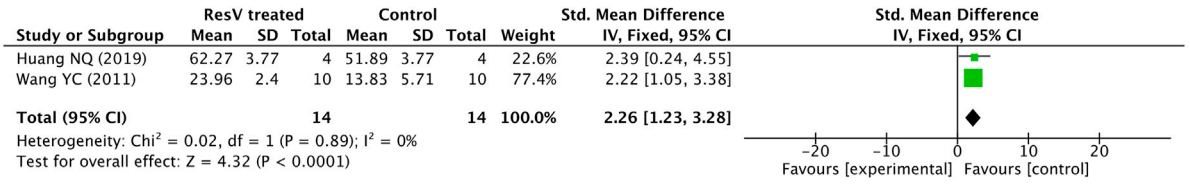

FIGURE 3 | Forest plot for TH$^+$ neuron counting measured by IHC in 6-OHDA–induced PD rats: resveratrol vs. vehicle control. The mean difference and standard error of TH$^+$ neuron counting in the resveratrol and vehicle-treated groups were quantificationally measured by ImageJ. These data were meta-analyzed in the form of forest plot by RevMan.

the selected literatures, one study performed the object recognition test and Y-maze test, and the results showed that resveratrol relieved cognitive deficiency in PD mouse model.

Neuropathological Analysis

For better neuropathological analysis on PD models, we selected the form of forest plot by RevMan to meta-analyze the MPTP and 6-OHDA–induced PD models. Because only 5 studies showed the TH$^+$ neuron counting numbers, we selected 3 studies which show TH$^+$ neuron counting measured by IHC in MPTP-induced PD mice. A total of 15 MPTP-induced PD mice were treated with resveratrol via different doses and for different lengths of time (50–100 mg/kg/day, for 13–33 days), at the same time, the control group with 15 mice was administered with the vehicle.

The meta-analysis outcome is displayed in the subsequent forest plot (**Figure 2**). The exploratory group was treated by resveratrol, the quantity of TH$^+$ neurons was distinctly upregulated when it was compared with the vehicle-administered group ($p < 0.00001$), with an average difference of 5.04. Furthermore, there was no heterogeneity in the outcome ($p = 0.52$, $I^2 = 0\%$).

In 2 studies, a total of fourteen 6-OHDA–induced PD rats received different doses of resveratrol for different periods (4 SD rats with 15 mg/kg/day for 36 days and 10 Wistar rats with 20 mg/kg/day for 14 days), and 14 rats were relatively administered with vehicles.

The forest plot outcome is shown in **Figure 3**, the quantity of TH$^+$ neurons were obviously increased in the resveratrol-treated group ($p < 0.0001$) in contrast with the vehicle group, with an average difference of 2.26. In addition, there was no heterogeneity in the total result ($p = 0.89$, $I^2 = 0\%$).

Neuroprotective Mechanisms Analysis

As is shown in **Figure 4**, a range of neuroprotective mechanisms for resveratrol were described in the selected studies. Most of the studies found that resveratrol ameliorated motor dysfunction, increased the level of dopamine and its metabolites, improved striatal TH protein levels, reduced the expression of α-synuclein, and improved the antioxidant status. Moreover, some studies illustrated that resveratrol reduced the neuroinflammatory reactions and regulated mitochondrial dysfunction.

Five studies reported the antioxidant effect of resveratrol. Anandhan et al. (2010) indicated that resveratrol improved the antioxidant function, enhanced the activity of glutathione (GSH) and glutathione peroxidase (GPx), as well as decreased superoxide dismutase (SOD) and catalase (CAT) levels. Khan et al. (2010) demonstrated that resveratrol enhanced the levels of glutathione reductase (GR), antioxidant enzymes (GPx, SOD, CAT), and protein carbonyl (PC). Its antioxidative activeness is the mechanism for its neuroprotective effects. Wang et al. (2011) indicated that resveratrol had antioxidant and radical scavenging ability, which resulted the protection of DA in PD rats. Mudo et al. (2012) showed that resveratrol elevated the antioxidants via increasing SOD2 and Trx2 levels in transgenic mice model. Palle et al. reported that resveratrol attenuated oxidative stress in rotenone-induced PD rat model.

Five literatures showed that resveratrol executed an anti-inflammatory function on the PD modes. Srivastava et al. (2012) indicated that resveratrol reduced microglial activation and neuroinflammation via increasing Cyp2d22 expression. Lofrumento et al. (2014) demonstrated that resveratrol sharply decreased glial activation, reduced TNF-αand IL-1β levels, enhanced TH-immunoreactivity, and increased the suppression of cytokine signaling-1 (SOCS-1). The mechanism

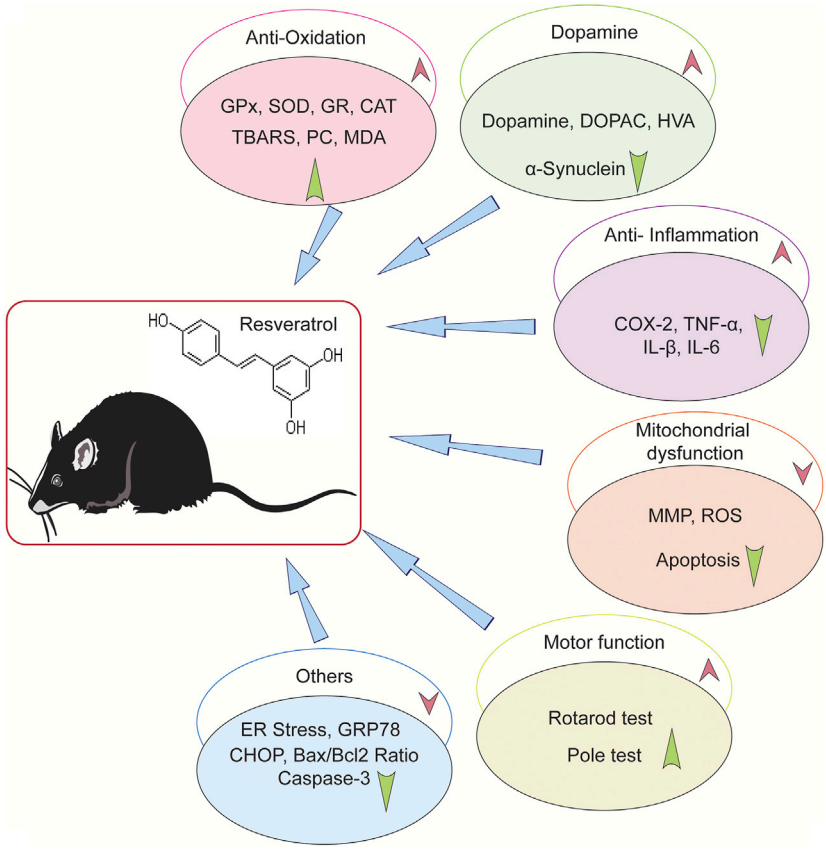

FIGURE 4 | Neuroprotective mechanisms of resveratrol in animal PD model. "↑" Means upregulation, "↓" means down-regulation.

may be related with its anti-inflammatory action by SOCS-1 induction (Lofrumento et al., 2014). Gaballah et al. (2016) and Zhang et al. (2018) showed that resveratrol ameliorated neuroinflammatory reaction. Liu et al. (2019) indicated that resveratrol decreased the α-synuclein in the striatum, increased the Bcl-2 level, reduced the caspase-3, Bax and IL-β levels, and improved the pAkt/Akt ratio. These effects enhanced striatum dopamine neuron survival.

Two studies show that resveratrol ameliorated mitochondrial dysfunction. Jin et al. (2008) indicated that resveratrol alleviated chromatin condensation and mitochondrial dysfunction, and it had neuroprotective effects related to anti-inflammation by declining COX-2 and TNF-α levels. Mudo et al. (2012) used a transgenic animal model to show that peroxisome proliferator-activated receptor-gamma coactivator-1α (PGC-1α) has a crucial role in oxidating stress and mitochondrial activity. The result showed that resveratrol had the same effect with overexpressing PGC-1α on protecting DA withstanding the MPTP-induced cell death. *In vitro* studies showed that resveratrol activated PGC-1α through deacetylating SIRT1, and increased SOD2 and Trx2 levels. Resveratrol played a neuroprotective role through the SIRT1/PGC-1α pathway.

Besides, Gaballah et al. (2016) reported resveratrol reduced ER stress through decreasing GRP78 and CHOP expression and inhibited the activity of caspase-3 in rat brain. Resveratrol also maintained the antioxidant status in the cell through activating the glutathione peroxidase and Nrf2 signaling pathway. Huang et al. (2019) reported that resveratrol reduced the 6-OHDA–caused apoptosis in Sprague–Dawley (SD) rats by motivating the phosphoinositide 3 kinase (PI3K)/protein kinase B (Akt) pathway and decreasing the Bax/Bcl-2 ratio and caspase-3 expression (Huang et al., 2019).

Methodological Quality Assessment

It is important to use the risk of bias as an evaluation index to evaluate the methodological quality in the experiment design and interpretation of results. Here, we employed the SYRCLE'S risk of bias tool to assess the technical value of 18 studies as stated in the guidance of Hooijmans et al., 2014 In **Table 3**, "+" means a minor extent of bias in the mark assessment and "–" represents a big chance of bias. "※" means the study did not include enough data to assess the degree of bias.

As shown in **Table 3**, in evaluating study quality on a scale of 1–10, the high score represents the high quality of the methodology in the articles. Most of the studies scored 2–6 in our validation. "Random allocation" was reported by 8 publications (44.4%); almost all the publications reported the "similar baseline features" (17 publications, 94.4%); for the "allocation concealment" and "blinded assessment of outcome," there was no publication reported the two

TABLE 3 | Methodological quality of studies.

Study	1	2	3	4	5	6	7	8	9	10	Score
Blanchet et al. (2008)	※	+	※	※	※	+	※	※	+	※	3
Jin et al. (2008)	※	+	※	+	※	+	※	−	+	※	4
Lu et al. (2008)	+	+	※	※	※	+	※	−	+	※	4
Anandhan et al. (2010)	※	+	※	+	※	※	※	−	+	※	3
Khan et al. (2010)	※	+	※	+	※	+	※	※	+	+	5
Wang et al. (2011)	+	+	※	※	※	+	※	※	+	+	5
Mudo et al. (2012)	※	+	※	※	※	+	※	※	+	※	3
Srivastava et al. (2012)	※	+	※	※	※	+	※	−	+	※	3
Lofrumento et al. (2014)	+	+	※	+	※	+	※	−	+	+	6
Wang et al. (2015)	※	+	※	※	※	※	※	−	+	※	2
Gaballah et al. (2016)	+	+	※	+	※	※	※	−	+	※	4
Guo et al. (2016)	+	+	※	+	※	+	※	−	+	※	5
Zhao et al. (2017)	+	+	※	+	※	+	※	−	+	※	5
Zhang et al. (2018)	※	※	※	+	※	+	※	−	+	※	3
Palle and Neerati, 2018	+	+	※	+	※	+	※	−	+	※	5
Huang et al. (2019)	+	+	※	+	※	+	※	※	+	※	5
Liu et al. (2019)	※	+	※	※	※	+	※	−	+	※	3
Xia et al. (2019)	※	+	※	※	※	+	※	※	+	※	3

1-stochastic distribution sequence; 2-analogous baseline traits; 3-distribution concealment; 4-stochastic housing; 5-blinded intervening; 6-random collection for outcome measurement; 7-blinded evaluation of result; 8-unfinished outcome data; 9-selecting outcome recording; 10-else sources of bias. +: yes; –: no; ※: unclear.

points; in addition, all the articles were "selective outcome reporting"; there were 15 articles (83.3%) that reported "random selection for outcome assessment"; 3 publications had other bias like analysis bias. Besides, sample size counting was not told in all the 18 articles; for animal experiment, sample size should be large enough to show a reasonable statistical significance yet small enough to avoid unnecessary use of animals.

DISCUSSION

Recently, a variety of natural compounds have been researched for their potentials to treat neurodegenerative diseases (Iyaswamy et al., 2020a; Iyaswamy et al., 2020b). Resveratrol is a small molecule which can be found in a range of common fruits and plants. Various studies show that resveratrol has neuroprotective effect (Jin et al., 2008; Lu et al., 2008; Della-Morte et al., 2009; Wang et al., 2015). The question is whether the results of these research studies are consistent and reliable.

The meta-analysis in this review employed an effective method to minimize the deviation of every study, which is very useful to assess the compound's effect in preclinical research. In this systematic review, we systematically evaluated the neuroprotective effect of resveratrol in various PD models to provide effective evidence for further application of resveratrol as an alternative medicine in PD clinical treatment.

In this review, we meta-analyzed the outcome data of TH^+ neuron counts of PD animal models by forest blot. The result showed that in the resveratrol administered group, the quantity of TH^+ neurons was distinctly upregulated when it was in contrast with the group treated with vehicle in MPTP-induced PD mice. Also, the quantity of TH^+ neurons was remarkably increased in the resveratrol administered group in contrast with the vehicle group in 6-OHDA–induced models.

This review assessed 18 published articles studying the effects of resveratrol on PD rodent models. Of the total 18 studies, 9 studies used MPTP-induced PD rodent models, 4 studies applied the 6-OHDA–lesioned PD models, 3 studies applied rotenone-induced PD models, 1 study used an A53T transgenic PD model, and 1 study used paraquat-induced PD models. In general, the studies showed that the protective mechanism of resveratrol is mainly involved in reducing the levels of α-synuclein and increasing TH protein levels. Some studies reported the changes in neuroinflammation, autophagy, and oxidative stress; a variety of signaling pathways, such as SIRT1/PGC-1α, PI3K/Akt, and MALATI/mir-129/SNCA, were also revealed. And for the 9 articles of MPTP-induced PD models, the neuroprotective effects of resveratrol were mainly due to antioxidation (3 articles) and anti-neuroinflammation (2 articles).

In summary, we systematically reviewed 18 studies evaluating the protective effectiveness of resveratrol in PD animal models, which were carefully screened from 3 databases. We have presented the effects of resveratrol and have discussed the mechanisms of action. The results of this study showed that resveratrol has obvious neuroprotective effects on different PD models via quantitative methods. The collective result in our review can supply useful information for researchers to further plan their future experiments. In addition, this collective evaluation of animal studies can supply a qualitative analysis of different PD animal models, either to guide further assessment of these models or to avoid needless duplication in their future research. Nevertheless, we feel that the meta-analysis outcomes supply sufficient proof for the ameliorative effect of resveratrol on PD animals to warrant further preclinical and clinical studies.

AUTHOR CONTRIBUTIONS

The manuscript was written by C-FS and LJ; the meta-analysis was assisted by X-WZ; the form analysis and the figure was done by C-FS, LJ, and AI; and the review was supervised and directed by ML.

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Efficacy and Safety of *Panax Notoginseng Saponins* (Xueshuantong) in Patients With Acute Ischemic Stroke (EXPECT) Trial: Rationale and Design

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Background: Although revascularization treatment is recommended as the first-line therapy for patients with non-minor acute ischemic stroke (AIS), it only benefits a minority of patients. Previous studies have reported the positive effects of *Panax notoginseng saponins* (PNS) (Xueshuantong lyophilized powder) on AIS, however, there have been no rigorous trials. This study aims to assess the efficacy and safety of PNS therapy for patients with AIS.

Methods: The Evaluation of Xueshuantong in Patients with acutE ischemiC sTroke (EXPECT) trial is a multicenter, randomized, placebo-controlled, double-blind study aiming to enroll 480 patients in China. Eligible patients with AIS within 72 h of symptom onset will randomly receive either PNS or PNS placebo for 10 days and subsequently be followed up to 90 days. The primary outcome will be a change in the National Institute of Health Stroke Scale (NIHSS) score from baseline to 10 post-randomization days. The secondary outcomes include early neurological improvement (proportion of patients with NIHSS score 0–1), and Patient-Reported Outcomes Scale for Stroke score at 10 post-randomization days, the proportion of patients with life independence (modified Rankin Scale score of 0–1), the proportion of patients with a favorable outcome (Barthel Index ≥90), and Stroke-Specific Quality of Life score at 90 days. Adverse events or clinically significant changes in vital signs and laboratory parameters, regardless of the severity, will be recorded during the trial to assess the safety of PNS.

Abbreviations: PNS, Panax Notoginseng Saponins; NIHSS, National Institute of Health Stroke Scale; PRO-Stroke, Patient-Reported Outcomes scale for Stroke; mRS, modified Rankin Scale; BI, Barthel Index; SS-QOL, Stroke-Specific Quality of Life; ICAM-1, intercellular adhesion molecule-1; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; MMP-9, matrix metalloproteinase-9.

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Conclusions: To our knowledge, this study is the first double-blind trial to assess the efficacy and safety of PNS in patients with AIS. Findings of the EXPECT trial will be valuable in improving evidence regarding the clinical application of PNS therapy in patients with AIS ineligible for revascularization treatment in the reperfusion era.

Keywords: acute ischemic stroke, panax notoginseng saponins, xueshuantong, efficacy, safety, randomized controlled trial

INTRODUCTION

Stroke is the second leading mortality cause worldwide and ranks first in China (Zhou et al., 2019; GBD 2019 Diseases and Injuries Collaborators, 2020). The high rates of stroke prevalence, incidence, and disability cause a significant economic burden (Rajsic et al., 2019; Wu et al., 2019a). Ischemic stroke is the most common stroke subtype, accounting for approximately 70% of all stroke cases (Wang et al., 2017). Currently, revascularization treatment within 24 h of symptom onset is recommended for saving the penumbra to improve functional outcomes in patients with non-minor acute ischemic stroke (AIS) (Powers et al., 2019). Since 1995, intravenous thrombolysis has been administered as the first-line therapy for AIS (NINDS, 1995). However, it benefits a limited number of patients given the narrow time-window, prevalent patient delay, imaging dependence, and risk of hemorrhagic transformation (Yaghi et al., 2017; Powers et al., 2019). Additionally, despite the extended time-window of endovascular thrombectomy, it has limited clinical application since it requires superior surgical skills, advanced catheter, rapid neuroimaging evaluation of the core infarction territory, and extensive economic resources (Report on Stroke Prevention and Treatment in China Writing Group, 2020). Patients with non-minor stroke presenting a National Institute of Health Stroke Scale (NIHSS) score higher or equal to four are likely to have unfavorable functional outcomes once they miss the critical treatment opportunity at the acute stage. Therefore, there is a substantial need to develop effective and safe therapies benefiting a large number of patients with AIS.

The pathophysiology of cerebral ischemic injury is a complex and dynamic process, during which, the temporal and spatial evolution of a rapid cascade of events including energy failure, excitotoxicity, oxidative and nitrative stress, and inflammatory response is associated with tissue damage following cerebral ischemia (Dirnagl et al., 1999; Lo et al., 2005; Chamorro et al., 2016). Among them, inflammatory injuries are triggered within minutes and last for weeks. Injured brain cells extensively produce pro-inflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), which results in neuronal damage. Consequently, there is an increased expression of adhesion molecules, including intercellular adhesion molecule 1 (ICAM-1), on the endothelial cell surface, which increases endothelial cell permeability, and therefore exacerbates ischemic injury (Barone et al., 1997; Dirnagl et al., 1999). Additionally, the accumulation of inflammatory mediators leads to blood-brain barrier disruption during the early phase after stroke onset (Brea et al., 2009; Giraud et al., 2015). As a result, leukocytes infiltrate the injured brain region and aggravate blood-brain barrier disruption in turn by releasing pro-inflammatory cytokines and matrix metalloproteinases (Neumann et al., 2015). Apart from focal inflammation, the so-called global inflammation responses occur and persist throughout the entire brain, affecting patients' clinical outcomes (Shi et al., 2019). Therefore, neuroinflammation is deemed as the potential treatment target (Jayaraj et al., 2019).

Panax notoginseng saponins (PNS) (Xueshuantong lyophilized powder) isolated from the roots and rhizomes of Panax notoginseng (Burkill) F.H.Chen consists of five main components: notoginsenoside R1, ginsenoside Rg1, ginsenoside Rd, ginsenoside Rb1, and ginsenoside Re (*see* Additional File 1 in **Supplementary Material**). The systematic pharmacokinetics of PNS indicates that the main circulating constituents are unchanged saponins, and intravenous PNS administration guarantees drug stability without inducing cytochrome P450 3A (Pintusophon et al., 2019; Zhang et al., 2020). PNS has been shown to exert strong anti-inflammatory effects against atherosclerosis-related cardiac-cerebral vascular disease (Wan et al., 2009; Wang et al., 2011). Both *in vitro* and *in vivo* studies have proved that PNS and notoginsenoside R1 significantly reduced the levels of IL-6, TNF-α, and ICAM-1 via microRNA downregulation, inhibiting NF-κB signaling pathway activation, and increasing the anti-inflammatory factor levels (Huang et al., 2015; Shi et al., 2017; Fu et al., 2018; Meng et al., 2019). Besides, PNS and ginsenoside Rb1 have been reported to attenuate ischemia-reperfusion-induced degradation of endothelial tight junctions by inhibiting matrix metalloproteinase-9 (MMP-9) expression and increasing the tissue inhibitor levels of metalloproteinase, which alleviates blood-brain barrier disruption (Chen et al., 2015; Wu et al., 2019b). Other studies have demonstrated the neuroprotective effects of PNS concerning antioxidant capacity (Zhang et al., 2019), anti-apoptosis (Chen et al., 2011), and endothelial cell protection (Hu et al., 2018).

PNS administration to patients with AIS within 72 h of symptom onset improves local brain perfusion and promotes the structural plasticity of white matter fibers (Gui et al., 2013; Ren et al., 2018). However, these findings were reported by small-scale, open-label, single-center studies, which limited the robustness of the conclusions. It remains unclear whether patients with non-minor stroke could benefit from PNS therapy. Therefore, there is a need for a large-scale, well-designed, randomized controlled trial with clinical endpoints to determine the effects of PNS on patients with AIS. We further hypothesize that short-term treatment with PNS for patients with AIS could effectively decrease the NIHSS score. We, therefore, designed the **E**valuation of **X**ueshuantong in

TABLE 1 | Inclusion and exclusion criteria of the EXPECT trial.

Inclusion criteria
Acute ischemic stroke confirmed by head CT or MRI
Female or male patient aged ≥18 years and ≤80 years
Time from symptom onset to the randomization ≤72 h
4 ≤ NIHSS score ≤16 (total score of upper and lower limbs on motor deficits ≥2) at the randomization time
Signed informed consent
Exclusion criteria
Having already received thrombolysis or endovascular treatment before randomization
Secondary stroke caused by a tumor, traumatic brain injury, hematological disease, or other diseases with a confirmed diagnosis
Preceding mRS score ≥2
Other conditions that cause motor dysfunction (claudication, severe osteoarthrosis, rheumatoid arthritis, gouty arthritis, etc.).
Known severe liver or kidney dysfunction
Known allergies for ingredients in the investigational product, allergy history for food or medicine
Known medical condition likely to limit survival to less than 3 months
Known massive cerebral infarction combined with disturbance of consciousness (1a ≥2 in the NIHSS), dementia, mental impairment, or unsuitability for participation as judged by the investigators
Pregnancy or breastfeeding
Having participated in another clinical trial within 3 months before randomization

NIHSS, National Institute of Health Stroke Scale; mRS, modified Rankin Scale.

Patients with acut**E** ischemi**C** s**T**roke (EXPECT) trial to assess the efficacy and safety of PNS in patients with AIS.

METHODS AND DESIGN

Study Design

The EXPECT trial (Clinicaltrials.gov, NCT04415164) is a prospective, multicenter, randomized, placebo-controlled, double-blind study to test the hypothesis that PNS is superior to placebo in decreasing the NIHSS score of patients with AIS after 10 days. This trial protocol was approved by the institutional review board of Dongzhimen Hospital, Beijing University of Chinese Medicine, Beijing, China (No. DZMEC-JG-2019-51-01). We described this protocol according to the SPIRIT 2013 Statement (Chan et al., 2013) and the complete checklist is available (*see* Additional File 2 in **Supplementary Material**).

Patient Selection

We will recruit patients diagnosed as AIS with an NIHSS score of 4–16 (a total score of upper and lower limbs ≥2 on motor deficits), who can be randomized within 72 h of symptom onset, which is defined based on the "last seen normal" principle. The age of recruited patients will be limited to 18–80 years. All patients or their legally authorized representatives will provide written informed consent before any study-specific procedure. **Table 1** lists the detailed inclusion and exclusion criteria.

Randomization, Allocation, and Blinding

The investigators will randomize 480 eligible patients and assign them to the intervention and control groups at a 1:1 ratio using block randomization with stratification according to medical centers. The randomization schedule will be generated by an independent statistician using SAS software version 9.4 (SAS Institute Inc.) and kept in sealed, sequentially numbered, opaque envelopes. Investigational medicine blinding will be completed at a pharmaceutical factory based on the randomization schedule and sent to medical centers along with the emergency envelopes. The block size will be closed to ensure concealment throughout the entire trial period. All investigators, participants, caregivers, and data analysts will be blinded to the treatment assignments throughout the trial until the blind codes are unconcealed. Investigators can request emergency unblinding in case of serious adverse events (SAEs) suspected to be associated with investigational medicine. **Figure 1** presents a flowchart of the EXPECT trial.

Treatment

Eligible patients will be assigned to the intervention and control groups. The intervention group will receive daily single infusions of 500 mg PNS diluted with 250 ml of 0.9% sodium chloride injection for 10 days. The control group will receive a PNS placebo using the aforementioned protocol. The PNS and PNS placebo will be manufactured and supplied by Guangxi Wuzhou Pharmaceutical (Group) Co., Ltd. with identical appearance, color, and flavor. All patients will receive current guideline-recommended standard treatments, including the basic control of risk factors and anti-platelets for AIS (Powers et al., 2019). **Figure 1** presents the treatment assignments. Edaravone and butylphthalide administration will be strictly prohibited during the treatment period. Investigational medicine will be discontinued in case of SAE occurrence, study withdrawal request from the patients or their legally authorized representatives, or poor compliance or non-adherence to the prescribed interventions. We will faithfully record the reasons for discontinuing interventions.

Study Settings and Recruitment Strategies

Inpatients will be recruited from 11 tertiary hospitals throughout eight provinces in China. Additionally, poster advertisements will be placed in these medical centers to allow the patients to

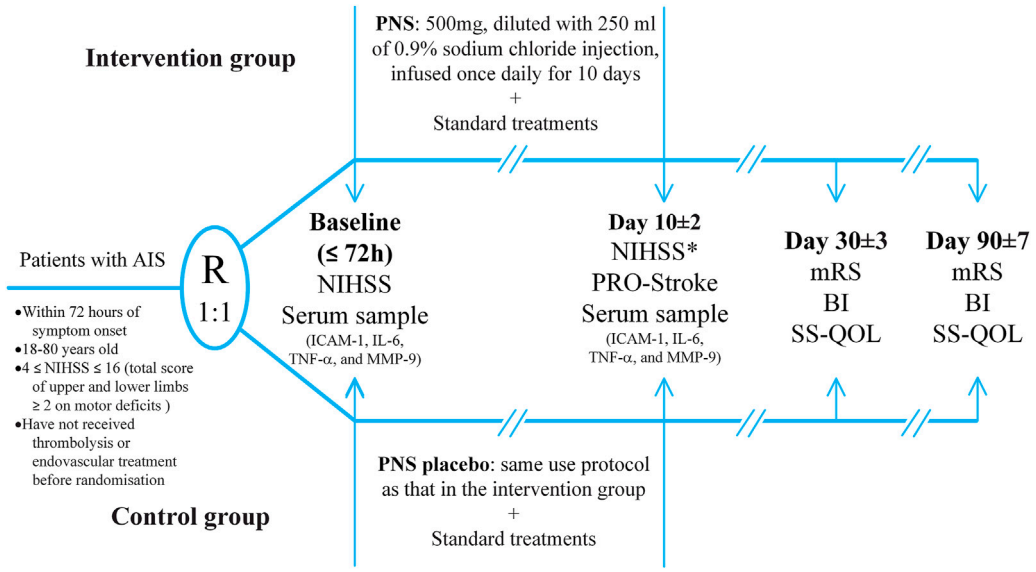

FIGURE 1 | The flowchart of the EXPECT trial.

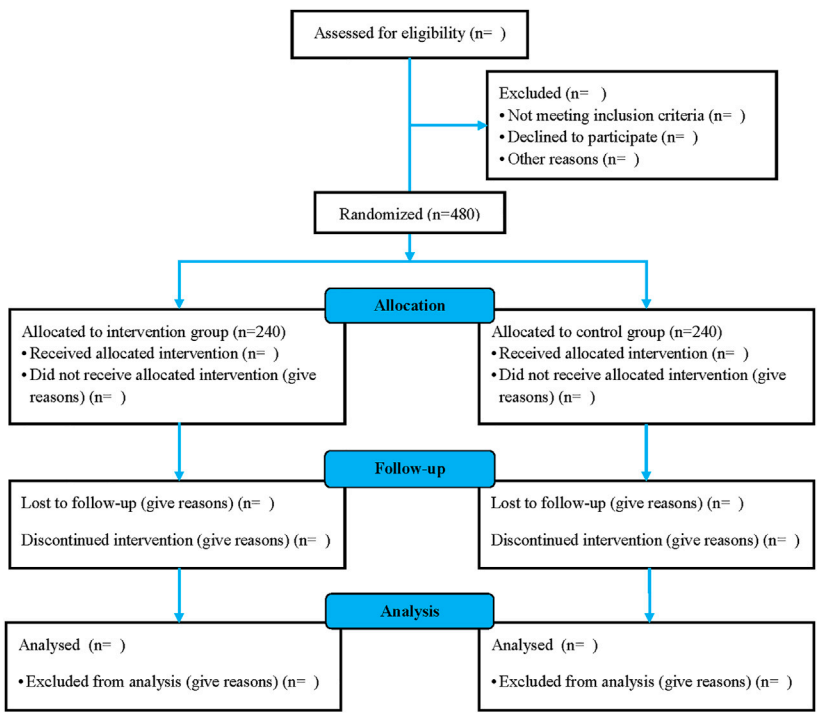

FIGURE 2 | The schematic diagram of the patient timeline.

voluntarily contact investigators. Potential patients will be screened for eligibility based on the inclusion and exclusion criteria. Eligible patients will be informed regarding the risks and benefits of the study. Subsequently, patients or their legally authorized representatives will sign the informed consent form if they agree to participate in the study. Patient enrollment of the EXPECT trial began in September 2020. Until December 2020, 13 patients had been enrolled and the estimated primary completion will be October 2022. **Figure 2** presents a schematic diagram of the patient timeline.

Efficacy Outcomes

In this trial, the primary outcome is neurological deficit amelioration defined as a change in the NIHSS score from baseline to 10 post-randomization days. Secondary outcomes will be as follows: 1) the proportion of patients with early neurological improvement (NIHSS score 0–1) at 10 post-randomization days; 2) patients' subjective feelings measured at 10 post-randomization days using the Patient-Reported Outcomes scale for Stroke (PRO-Stroke), which is a well-validated scale suitable in Chinese patients for assessing stroke and treatment effects on physical function, psychological change, social engagement, and treatment satisfaction (Wang et al., 2012a; Wang et al., 2012b; Wang et al., 2012c); 3) proportion of patients with life independence (90-days modified Rankin Scale [mRS] score ≤1); 4) proportion of patients with a favorable outcome (90 days BI score ≥90); and 5) patients' quality of life measured using the Stroke-Specific Quality of Life (SS-QOL) at 90 days.

Biological Outcomes

We will perform between-group comparisons of the changes in ICAM-1, IL-6, TNF-α, and MMP-9 levels from baseline to 10 post-randomization days.

Safety Outcomes

The safety outcome will include any adverse events (AEs), SAEs, and clinically meaningful changes in vital signs or laboratory parameters during the trial period.

Follow-Up Procedures

The EXPECT trial contains four visits including time at randomization (baseline), 10 ± 2 days after randomization, 30 ± 3 days, and 90 ± 7 days after stroke onset. At baseline, we will evaluate demographic characteristics, routine laboratory tests, non-contrast CT/MRI, vessel imaging (carotid artery ultrasound/transcranial Doppler imaging/MR angiography), electrocardiogram (ECG), and NIHSS. At 10 ± 2 days, we will perform assessments using the PRO-Stroke, repeated routine laboratory tests, ECG, and NIHSS. Biological samples will be collected at both baseline and 10 ± 2 days. The mRS, BI, and SS-QOL scores will be determined at 30 ± 3 days and 90 ± 7 days. Finally, vital signs and complications will be recorded at these four visits; on the other hand, AEs and SAEs will be recorded at any time during the trial.

Data Collection and Management

Data collection and management will be performed in collaboration with clinical doctors and clinical research coordinators. All investigators in charge of patient recruitment, outcome assessment, data collection, and serum sample collection will receive pre-recruitment standardized training regarding this trial's standard operating procedures. Investigators in all medical centers will make a reasonable effort to follow-up with the patient throughout the study period. Information obtained from patients will be recorded in the investigative case form by investigators; subsequently, the clinical research coordinator will perform data entry into electronic case report forms using a unique login ID. All patient-related information will be stored in locked file cabinets with limited access at medical centers. All serum samples, reports, data collection, and administrative forms will be only identified using a coded ID number to maintain participant confidentiality.

Quality Control and Data Monitoring

The Steering Committee will be responsible for the scientific content of the protocol, overseeing the study operations, supervising the intra-study data sharing process, and preparing the primary manuscript and other publications arising from the EXPECT trial. Two contract research organizations will regularly perform data monitoring and data quality control. Data analysis will be completed by a third-party statistical unit (Tianjin University of Traditional Chinese Medicine).

Adverse Events Management

All AEs will be evaluated for their association with investigational medicine; subsequently, they will be treated, recorded, and followed-up until recovery or stabilization. The investigators will report any SAE to the ethics committee, contract research organization, principal investigator, and China Food and Drug Administration.

Sample Size Calculations

Based on a previous study that reported a mean decrease in the NIHSS score of 3 and 2.1 in the PNS and control groups, respectively, and a standard deviation of 2.5 (Wang, 2009), this trial will require 480 patients with a power of 90%, two-sided α of 0.05, and a 20% dropout rate to test the hypothesis that PNS is superior to placebo in decreasing the NIHSS score at 10 post-randomization days in patients with AIS.

Statistical Analysis Plan

All randomized patients who receive at least one treatment dose and have safety outcome data will be included in the safety set. All patients in the safety set will be included in the full analysis set if efficacy outcome data are available. All patients in the full analysis set who are deemed to have no major protocol violations will be included in the per-protocol set. Efficacy and safety analyses will be performed according to the intention-to-treat principle. Additionally, per-protocol data analysis will be conducted as a reference. The last observation carried forward approach will be used to impute missing data of primary outcome. Regarding the primary outcome variable, between-group comparisons of the change in NIHSS score will be performed using Student's *t*-test or Mann-Whitney *U* test, as appropriate. Regarding secondary outcome variables, between-group comparisons of the proportion of patients with an NIHSS score of 0–1, mRS grade ≤1, and BI score ≥90 will be performed using the chi-square test or Fisher exact test. Further between-group comparisons of the PRO-Stroke and SS-QOL score, as well as changes in biological indexes, will be compared using the *t*-test or Mann-Whitney *U* test. Moreover, between-group comparisons of the incidence of AEs will be compared using the chi-square or

Fisher exact test. The effect of missing data on the results will be assessed through sensitivity analysis. All statistics will be 2-sided and statistical significance will be set at $p < 0.05$. Statistical analyses will be performed using SAS software version 9.4 (SAS Institute Inc.).

Subgroup Analyses

Subgroup analyses for the primary outcome will be performed according to the following baseline characteristics: age (> 65 years vs. ≤ 65 years); gender (female vs. male); symptom onset to randomization time (≤24 h vs. > 24 h); disease history of hypertension, diabetes mellitus, coronary heart disease, stroke, and hypercholesterolemia; smoking history; Trial of Org 10,172 in Acute Stroke Treatment classification; main arterial stenosis; and stroke severity based on the NIHSS score.

DISCUSSION

Given that only a minority of patients with non-minor AIS could benefit from revascularization treatment, there remains a need for safe pharmacological neuroprotection against brain tissue injury in AIS treatment. Previous unsuccessful translational research on neuroprotective agents with unimodal targets has indicated the need for a single medicine blocking different key AIS-related mechanisms based on the complex pathophysiological cascade events of AIS (Rogalewski et al., 2006). Preclinical studies have reported the positive effects of PNS in alleviating inflammation injuries, anti-oxidation, and anti-apoptosis (Wan et al., 2009; Chen et al., 2011; Wang et al., 2011; Chen et al., 2015; Huang et al., 2015; Shi et al., 2017; Fu et al., 2018; Meng et al., 2019; Wu et al., 2019a; Zhang et al., 2019). Notably, exploratory studies using neuroimaging examination as the surrogate endpoint have demonstrated the therapeutic effect of PNS (Gui et al., 2013; Ren et al., 2018). However, the precise effect of PNS on patients with AIS should be further assessed using clinical endpoints.

As one of the main considerations in a trial design, the selection of an appropriate primary outcome is largely dependent on the disease and should reflect the treatment effect and expected mechanism. In this EXPECT trial, the post-intervention neurological improvement according to the NIHSS score is the primary clinical endpoint. The short time-span between treatment and NIHSS assessment requires relatively less effort to trace patients meanwhile reducing the risk of loss to follow-up. Researchers performed a causal mediation model using combined data from the MR CLEAN trial and IMS III trial and found that the change of NIHSS score reflected the treatment effect and lay on the causal pathway between treatment and long-term mRS categories. The results suggested that the NIHSS measures both neurological deficits and functional outcomes and that it could act as an alternative primary outcome for AIS treatment trials (Chalos et al., 2020).

To our knowledge, the EXPECT trial is the first multicenter, randomized, double-blind, placebo-controlled trial to assess the efficacy and safety of PNS in patients with AIS in the reperfusion era. This study is limited in terms of the lack of imaging assessment in follow-up procedures as unfavorable outcome predictors due to inadequate funding. However, we will explore the therapeutic mechanism of PNS therapy in the alleviation of cerebral ischemia-induced inflammatory damage. Moreover, the results will be valuable to interpret the efficacy of PNS. In summary, the EXPECT trial will provide critical evidence for PNS therapy for the vast majority of patients with AIS who are ineligible or have missed the opportunity for revascularization treatment.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Dongzhimen Hospital, Beijing University of Chinese Medicine (No. DZMEC-JG-2019-51-01). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YG proposed the conception of the trial. FH, LZ, SW, YD, and YG designed the trial and oversaw all of the scientific aspects regarding its implementation. LF drafted the manuscript. DZ and CZ revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.648921/full#supplementary-material.

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REVIEW
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Ferroptosis as a New Mechanism in Parkinson's Disease Therapy Using Traditional Chinese Medicine

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Parkinson's disease (PD) is one of the most common neurodegenerative diseases. To date, among medications used to treat PD, only levodopa exhibits a limited disease-modifying effect on early-onset PD, but it cannot delay the progression of the disease. In 2018, for the first time, the World Health Organization included traditional Chinese medicine (TCM) in its influential global medical compendium. The use of TCM in the treatment of PD has a long history. At present, TCM can help treat and prevent PD. Iron metabolism is closely associated with PD. Ferroptosis, which is characterized by the accumulation of lipid peroxides, is a recently discovered form of iron-dependent cell death. The research literature indicates that ferroptosis in dopaminergic neurons is an important pathogenetic mechanism of PD. TCM may thus play unique roles in the treatment of PD and provide new ideas for the treatment of PD by regulating pathways associated with ferroptosis.

Keywords: Parkinson's disease, traditional Chinese medicine, iron metabolism, ferroptosis, mechanism of ferroptosis

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease and is second only to Alzheimer's disease. The death toll due to PD increased by 42.4% between 2005 and 2015 (GBD, 2016). According to pathological studies, a reduction in the number of dopaminergic neurons and an abnormal accumulation of α-synuclein (α-syn) result in a shortage of dopamine from the substantia nigra–striatum pathway, causing clinical symptoms, such as tremor, bradykinesia, rigidity, and postural balance disorders (Burbulla et al., 2017; Mittal et al., 2017). The brain is the main tissue in which iron accumulates (Belaidi and Bush, 2016). The development of PD is closely associated with the metabolism and homeostasis of iron in the brain tissue. Ferroptosis is a recently identified iron-dependent cell death pathway that is triggered by a buildup of lipid peroxides and is different from apoptosis (Tiepolt et al., 2018). A lethal buildup of lipid reactive oxygen species (ROS) and an imbalance in oxidation–reduction reactions lead to ferroptotic cell death. Ferroptosis is associated with neurodegenerative disorders, tumors, and cardiovascular diseases. In recent years, ferroptosis has been associated with the pathogenic changes observed in PD.

The goal of the treatment of PD is to relieve symptoms, including motor symptoms and non-motor symptoms, for instance, emotional disorder and pain, by increasing the striatal level of dopamine (Demaagd and philip, 2015). Levodopa is the main treatment for symptoms of PD, although Verschuur et al. (2019) concluded that in patients with early PD treatment with levodopa, it had no disease-modifying effect. However, de Bie et al. (2020) considered that until more effective methods providing

TABLE 1 | Metabolic pathways in ferroptosis.

Metabolic pathway	Main pathway	Metabolite(s)	References
Iron metabolism	Fenton reaction/Haber–Weiss reaction	Fe^{2+}	Yan and Zhang (2019); Lei et al. (2019)
Amino acid metabolism	System Xc^-/GPx4	Glutathione disulphide	Dixon et al. (2012); Friedmann Angeli et al. (2014)
Lipid metabolism	Polyunsaturated fatty acids	Phosphatidylethanolamine-arachidonic acid/ adrenic acid	Stockwell et al. (2017); Dixon et al. (2012)
Coenzyme Q10 biosynthesis	Ferroptosis suppressor protein 1/mevalonate pathway	Reduced coenzyme Q10	Doll et al. (2019); Bersuker et al. (2019)

GPx4, glutathione peroxidase 4

stable dopamine concentrations are developed, current evidence supports the use of levodopa as the initial symptomatic treatment in most patients with PD. The use of traditional Chinese medicine (TCM) in the treatment of PD has a long history. In 2018, for the first time, the World Health Organization included TCM in its influential global medical compendium. The treatment of PD using TCM was described in ancient Chinese medicinal texts, for example, in Huangdi Neijing (425–221 BC), the tremor and stiffness are described as "Chan, Chi, Jing." Later works supplemented this in terms of the preventive treatment and experiences of alleviating the symptoms of PD. In recent years, the results of numerous studies showed that Chinese medicine, Chinese medicine compounds, and single extracts have effects on the regulation of ferroptosis.

FERROPTOSIS AND ASSOCIATED PATHWAYS

Ferroptosis

Ferroptosis is a phenomenon that involves regulated cell necrosis caused by lipid peroxidation induced by iron and ROS (Dixon et al., 2012). The main cellular metabolic mechanisms in ferroptosis include iron metabolism, amino acid metabolism, and lipid metabolism (Friedmann Angeli et al., 2014; Stockwell et al., 2017; Yan and Zhang, 2019). The signaling pathway mediated by glutathione peroxidase 4 (GPx4) (Friedmann Angeli et al., 2014; Yang et al., 2014) and radical-trapping antioxidants (Zilka et al., 2017; Shah et al., 2018) is a classic signal regulation pathway of ferroptosis. Recently, scientists have discovered that the expression of the ferroptosis suppressor protein 1 in cells can significantly protect cells from the adverse effects of factors that induce ferroptosis. Clearly, coenzyme Q10 produced *via* the mevalonate pathway has an antioxidant function in cells, for which it acts as an endogenous inhibitor of ferroptosis by preventing lipid oxidation (Bersuker et al., 2019; Doll et al., 2019) (see **Table 1**).

Mechanism of Ferroptotic Cell Metabolism

Iron metabolism is one of the main mechanisms of ferroptotic cell metabolism. An excess of iron ions in cells can produce lipoxygenase, which causes lipid peroxidation and thus leads to ferroptotic cell death. Excessive amounts of Fe^{2+} in cells generate lipid ROS through the Fenton reaction or Haber–Weiss reaction and thus oxidize cell membrane lipids. Moreover, Fe^{2+} is also an important part of the catalytic subunit of lipoxygenase (Lei et al., 2019). Heme oxygenase-1 is a critical enzyme in heme metabolism and decomposes heme into Fe^{2+} and biliverdin. Heme metabolism, which is an important source of intracellular iron, plays a key role in erastin-induced ferroptotic cell death, such as inducing lipid peroxidation (Kwon et al., 2015). Amino acid metabolism is another crucial metabolic mechanism of ferroptotic cell death because it inhibits the synthesis of glutathione (GSH). System Xc^- in the cell membrane transports extracellular cystine and intracellular glutamate in a ratio of 1:1 (Dixon et al., 2012). The intracellular concentration of glutamate is high, and the difference between its concentrations inside and outside the cell is the driving force for its transport. When the neuronal cells in the brain tissue are damaged, system Xc^- will be inhibited by a high concentration of extracellular glutamate (Bridges et al., 2012; Friedmann Angeli et al., 2014). Cystine is transported into the cell and then decomposed into cysteine. Then, γ-glutamylcysteine is generated with glutamic acid under the catalytic action of glutamylcysteine ligase. Subsequently, the reaction of γ-glutamylcysteine with glycine is catalyzed by glutathione synthase, which generates GSH (Aoyama and Nakaki, 2015). GPx4 is a key enzyme involved in lipid peroxidation in amino acid metabolism in the metabolic mechanism of ferroptosis, and it can degrade small-molecule peroxides and certain lipid peroxides and inhibit lipid peroxidation (Stockwell et al., 2017). Erastin, which is an inducer of ferroptotic cell death, can hinder the synthesis of GSH by inhibiting the cystine–glutamate exchanger in the plasma membrane and reducing the cellular uptake of cysteine. GSH is necessary for GPx4 lipid repair activity. When GSH is consumed, GPx4 is inactivated, which in turn causes the accumulation of membrane lipid ROS and ferroptosis (Friedmann Angeli and Conrad, 2018). Abnormal lipid metabolism may also lead to ferroptosis (**Figure 1**). The contents and subcellular locations of amino acids and polyunsaturated fatty acids determine the degree of ferroptosis (Stockwell et al., 2017). Polyunsaturated fatty acids and membrane phospholipids generate arachidonic acid and adrenic acid *via* the esterification of phosphatidylethanolamine (Shindou and Shimizu, 2009), and arachidonic acid and adrenic acid are the main substrates of lipid peroxidation in ferroptotic cell death (Yan and Zhang, 2019).

FERROPTOSIS AND PARKINSON'S DISEASE

Iron and Parkinson's Disease

Iron is required for synaptic growth, the formation of myelin sheaths, and the production and transmission activity of

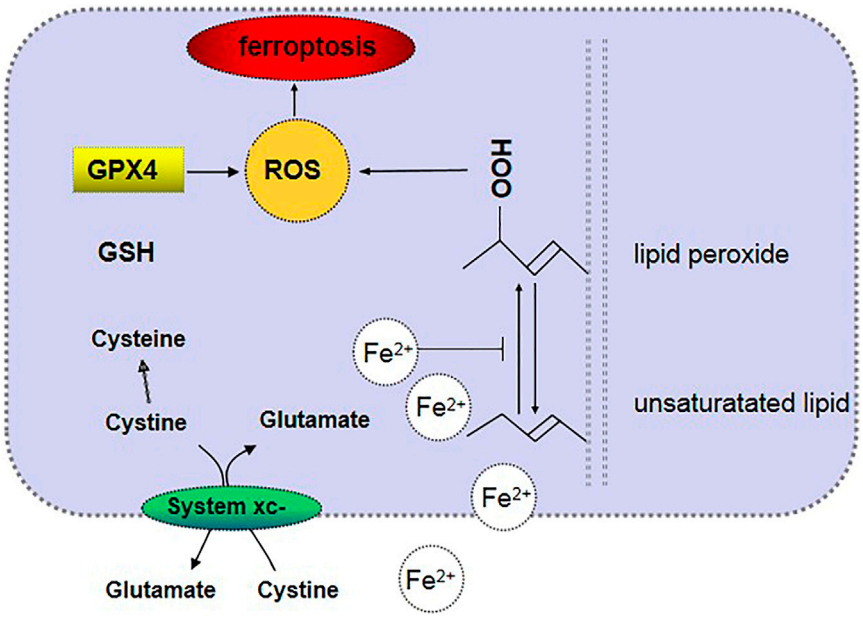

FIGURE 1 | Mechanism of ferroptosis.

neurotransmitters, and is also essential for normal brain development and cognitive functioning (Tiepolt et al., 2018). Iron progressively accumulates in neurodegenerative disorders with age (Abeyawardhane et al., 2018), with greater accumulation observed in the brain, including the nigrostriatal pathway and basal ganglia, namely, the globus pallidus and dopaminergic neurons in the caudate nucleus and putamen. According to pathological studies, a reduction in the number of dopaminergic neurons and an abnormal accumulation of α-syn result in a shortage of dopamine in the substantia nigra–striatum pathway, which causes clinical symptoms, such as tremor, bradykinesia, rigidity, and postural balance disorders. PD is associated with iron, which is particularly abundant in the dopaminergic neurons in the substantia nigra pars compacta (Chmatalova et al., 2017) as an integral component of tyrosine hydroxylase–dependent dopamine synthesis, as well as other enzymatic and nonenzymatic reactions associated with dopamine metabolism (Do Van et al., 2016; Belarbi et al., 2017). Moreover, iron is correlated with an increased risk of the formation of α-syn fibers, which contributes to PD (Ito et al., 2017). The pathology of PD is associated with phospholipid oxidation and changes in iron-regulating mechanisms. Large amounts of Fe^{2+} in organisms are stored in the form of ferritin, whereas the rest of Fe^{2+} is transported through the blood to the cells, which is facilitated by transferrin. The conversion of divalent Fe^{2+} to Fe^{3+} occurs with the reduction of α-syn, and then Fe^{3+} combines with α-syn/C (Duce et al., 2017). The affinity of Fe^{2+} and Fe^{3+} for α-syn is very high, which could accelerate the accumulation of α-syn. The iron chelator, for example, desferrioxamine, can inhibit the abnormal folding of α-syn (Davies et al., 2011). Moreover, ferroportin is the only protein that transports iron into the extracellular space. When iron stored by macromolecules, such as neuromelanin and transferrin, cannot be stored stably, this iron accumulates in cells, and, under its catalytic action, phospholipid hydroperoxidation is induced. This then leads to the damage of dopaminergic neurons, decrease in the production of dopamine, and the disruption of the integrity of the iron metabolism pathway. The accumulation of iron in neurons and glial cells is associated with neurodegenerative disorders (**Figure 2**). The levels of accumulated iron reflect the clinical status of the disorders (Pyatigorskaya et al., 2015). Astrocytes are essential supporting cells in the neurovascular unit and serve to release iron supplied by the brain capillary endothelial cells to neurons while mitigating its toxicity (Burkhart et al., 2016). To this end, astrocytes are specialized for the export of iron.

Ferroptosis and Parkinson's Disease

Ferroptosis is a recently discovered pathogenetic mechanism of PD. Studies have found an increase in iron deposits (Lei et al., 2012; Do Van et al., 2016; Guiney et al., 2017), depletion of GSH (Sian et al., 1994), and lipid peroxidation (Dexter et al., 1989) in the substantia nigra pars compacta. Studies have also found that ferroptosis is involved in the development of MPTP-induced PD in mouse models. Moreover, the administration of the ferroptosis inhibitor ferrostatin-1 can reduce the damage to dopaminergic neurons in models of MPTP- and rotenone-induced PD (Do Van et al., 2016). In neurofibroma cells treated with MPP+, it was found that the inhibition of transferrin receptor 1 can reduce the aggregation of α-syn. Transferrin receptor 1 is an important upregulated protein in ferroptotic cells (Kalivendi et al., 2004). In patients with early PD, treatment with deferiprone can reduce oxidative stress and increase dopamine activity to slow the progression of motor deficits and alleviate existing motor

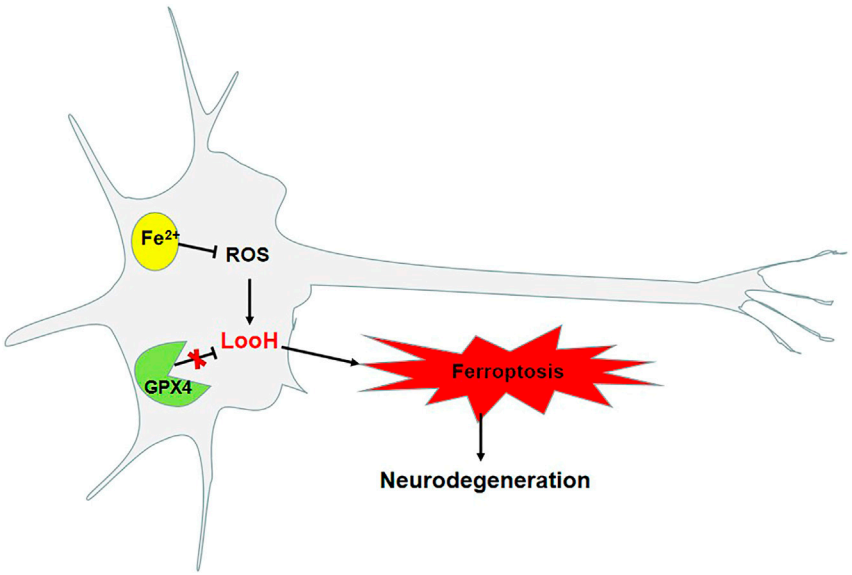

FIGURE 2 | Ferroptosis as a pathway in Parkinson's disease.

symptoms (Abeyawardhane et al., 2018). In a mouse model of MPTP-induced PD and a model of rotenone-induced cell injury, the administration of the ferroptosis inhibitor ferrostatin-1 can reduce the occurrence of oxidative stress, the deposition of excessive ROS, and the accumulation of α-syn in the substantia nigra and striatum. However, the administration of the ferroptosis inducer erastin can induce the occurrence of ferroptotic cell death, increase the abnormal accumulation of α-syn in mice, and decrease the number of dopaminergic neurons (Friedmann Angeli et al., 2014; Ito et al., 2017).

TCM IN TREATMENT OF PARKINSON'S DISEASE

At present, only a small proportion of the drugs that have a therapeutic effect on PD, such as levodopa, can treat patients with early PD, but these cannot delay the progression of the disease (de Bie et al., 2020). TCM has a long history. PD is classified as "tremor syndrome" in TCM, and Parkinson's disease–like symptoms have been described at length in ancient Chinese medicine books. PD was described as a disease with the tremor and stiffness in the first medical monograph, namely, *Huangdi Neijing*, more than 2,000 years ago. TCM has obvious curative effects in both the prevention and treatment of diseases by methods, such as formulas (**Table 2**), acupuncture, and exercises. The Eastern Han dynasty text *Shanghan Zabing Lun* proposed the use of the Zhenwu decoction and Gegen decoction for treating tremors. Later on, physicians have proposed the use of Fangfeng Tongsheng San (1115–1368 AD) and Ding Zhen Wan (1368–1644 AD) for treating Parkinson-related symptoms. At present, many treatment methods continue to be used in later generations and play a beneficial role in the prevention and treatment of PD. In *Huangdi Neijing*, the idea of disease prevention was emphasized. This view is consistent with the early diagnosis and treatment of the prodromal stage of PD, which is currently investigated by medical scientists. The non-motor symptoms of PD, such as rapid eye movement sleep behavior disorder, hypotension, depression, hyposmia, and constipation, appear 5–20 years earlier than the motor symptoms. Preventive intervention in PD during the prodromal period before motor symptoms develop may effectively delay or even reverse the development of the disease. TCM has obvious advantages in the prevention of diseases through overall adjustment.

TCM and Western medicine both have their own advantages in the treatment of PD. Part of researches considered Chinese medicine compounds containing medicine ingredients together with Western medicines were superior to single Western medicines in treating PD (Wu et al., 2018). The combination of TCM and Western medicine in the treatment of PD can increase the release of dopamine and improve sensitivity to dopamine, which will relieve the symptoms in patients (Kim et al., 2019). TCM preparations can effectively alleviate the motor symptoms of PD patients and improve their quality of life. Moreover, they can alleviate nausea and vomiting caused by Madopar, the "end-of-dose phenomenon," the "on–off phenomenon," and mental disorders (Shantian, 1997; Chen et al., 2012; Zhang et al., 2016). On-medicinal treatments, such as exercises and acupuncture, are beneficial in treating the motor non-motor symptoms of PD. Studies have found that Tai Chi can effectively improve patients' flexibility, such as their stride length and pace, increase their endurance, enhance their control of their posture and direction, and prevent falls. In fact, Tai Chi plays a useful role in improving the balance and functional ability of patients with early PD and can relieve

TABLE 2 | Classic traditional Chinese medicines with potential benefits for treating Parkinson's disease–associated symptoms.

Period	Text	Prescription	Decoction composition	Symptom(s)
Han dynasty (206 BC–220 AD)	Shanghan Zabing Lun	Zhenwu decoction	*Smilax glabra* Roxb. (Smilacaceae; Smilax rhizome), *Paeonia lactiflora* Pall. (Paeoniaceae; Paeonia root), *Zingiber zerumbet* (L.) Roscoe ex Sm. (Zingiberaceae; Zingiber rhizomes et root), *Aconitum carmichaelii* Debeaux (Ranunculaceae; Aconitum root tuber), *Atractylodes macrocephala* Koidz. (Asteraceae; Atractylodes rhizome)	Tremor
		Gegen decoction	*Pueraria montana* var. lobata (Willd.) Maesen and S.M.Almeida ex Sanjappa and Predeep (Fabaceae; Pueraria radix et root), *Ephedra sinica* Stapf (Ephedraceae; Ephedra root and rhizome), *Cinnamomum verum* J.Presl (Lauraceae; Cinnamomum terminal branchlet), *Paeonia lactiflora* Pall. (Paeoniaceae; Paeonia root), *Glycyrrhiza glabra* L. (Fabaceae; Glycyrrhiza radix et rhizome), *Zingiber zerumbet* (L.) Roscoe ex Sm. (Zingiberaceae; Zingiber rhizomes et root)	
	Huatuo Shen Shu	Fengshen decoction	*Saposhnikovia divaricata* (Turcz. ex Ledeb.) Schischk. (Apiaceae; Saposhnikovia rhizome), *Hansenia weberbaueriana* (Fedde ex H.Wolff) Pimenov and Kljuykov (Apiaceae; Hansenia radix et rhizome), *Eleutherococcus senticosus* (Rupr. and Maxim.) Maxim. (Araliaceae; Eleutherococcus radix et rhizome), *Paeonia lactiflora* Pall. (Paeoniaceae; Paeonia Pall root), *Panax ginseng* C.A.Mey. (Araliaceae; Panax radix et rhizome), *Coix lacryma-jobi* var. *ma-yuen* (Rom.Caill.) Stapf (Poaceae; Coix ripe kernel), *Scrophularia ningpoensis* Hemsl. (Scrophulariaceae; Scrophularia root), *Ophiopogon japonicus* (Thunb.) Ker Gawl. (Asparagaceae; Ophiopogon radix), *Rehmannia glutinosa* (Gaertn.) DC. (Orobanchaceae; Rehmannia rhizome and root), *Aucklandia costus* Falc. (Asteraceae; Aucklandia radix), *Pinus pinea* L. (Pinaceae; Pinus seed), magnetitum, *Areca catechu* L. (Arecaceae; Areca nut), *Citrus* × *aurantium* L. (Rutaceae; Citrus fruit), *Achyranthes bidentata* Blume (Amaranthaceae; Achyranthes radix), *Smilax glabra* Roxb. (Smilacaceae; Smilax rhizome), *Cinnamomum verum J.*Presl (Lauraceae; Cinnamomum terminal branchlet)	Spasm and tremor
Tang dynasty (618–907 AD)	Qianjin Yaofang	Bailian decoction	*Ampelopsis japonica* (Thunb.) Makino (Vitaceae; Ampelopsis root), *Zingiber officinale* Roscoe (Zingiberaceae; Zingiber dried rhizome), *Coix lacryma-jobi* var. *ma-yuen* (Rom.Caill.) Stapf (Poaceae; Coix ripe kernel), *Ziziphus jujuba* Mill. (Rhamnaceae; Ziziphus ripe kernel), *Achyranthes bidentata* Blume (Amaranthaceae; Achyranthes radix), *Cinnamomum verum* J.Presl (Lauraceae; Cinnamomum inner bark devoid of cork), *Paeonia lactiflora* Pall. (Paeoniaceae; Paeonia Pall root), *Plantago asiatica* L. (Plantaginaceae; ripe seed), *Glycyrrhiza glabra* L. (Fabaceae; Glycyrrhiza radix et rhizome), *Aconitum carmichaeli* Debeaux (Ranunculaceae; Aconitum root tuber)	Dystonia
Song dynasty (960–1279 AD)	Taiping Huiming Heji Ju Fang	Shexiang Tianma Wan	*Gastrodia elata* Blume (Orchidaceae; Gastrodia rhizome), *Conioselinum anthriscoides* 'Chuanxiong' (Apiaceae; Conioselinum rhizome), *Saposhnikovia divaricata* (Turcz. ex Ledeb.) Schischk. (Apiaceae; Saposhnikovia rhizome),*Chrysanthemum* × *morifolium* (Ramat.) Hemsl. (Asteraceae; Chrysanthemum flower), moschus, *Arisaema erubescens* (Wall.) Schott (Araceae; Arisaema tuber)	Tremor and dystonia
	Yangshi Jiacang Fang	Xunluo Wan	*Commiphora myrrha* (T.Nees) Engl. (Burseraceae; Commiphora oleo gum resin), *Boswellia carteri* Birdw. (Burseraceae; Boswellia resin exuding from the bark), Tiger bone, *Chinemys reevesii* (Gray) (Testudinidae; Chinemys carapace), *Angelica sinensis* (Oliv.) Diels (Apiaceae; Angelica radix and rhizome), *Trogopterus xanthipes* Milne-Edwards (Petauristidae; Trogopterus droppings), *Aconitum carmichaeli* Debeaux (Ranunculaceae; Aconitum root), *Gastrodia elata* Blume (Orchidaceae; Gastrodia rhizome), *Buthus martensii* Karsch (Scorpio; Buthus dried), *Arisaema erubescens* (Wall.) Schott (Araceae; Arisaema tuber), *Aconitum carmichaeli* Debeaux (Ranunculaceae; Aconitum rhizome), *Eucommia ulmoides* Oliv. (Eucommiaceae; Eucommia bark), *Pheretima pectinifera* Michaelsen (Lumbricus; Pheretima dried), *Clematis chinensis* Osbeck (Ranunculaceae; Clematis radix and rhizome), *Achyranthes bidentata* Blume (Amaranthaceae; Achyranthes radix), *Dipsacus asper* Wall. ex DC. (Caprifoliaceae; Dipsacus root), *Zaocys dhumnades* Cantor (Colubridae; Zaocys dried), *Cistanche deserticola* Ma (Orobanchaceae; Cistanche stem), Cinnabaris	Spasm and difficulty walking
Jin-Yuan dynasty (1115–1368 AD)	Xuanming Lunfang	Fangfeng Tongsheng decoction	*Saposhnikovia divaricata* (Turcz. ex Ledeb.) Schischk. (Apiaceae; Saposhnikovia rhizome), *Rheum officinale* Baill. (Polygonaceae; Rheum radix et rhizome), *Conioselinum anthriscoides* 'Chuanxiong' (Apiaceae; Conioselinum rhizome), *Angelica sinensis* (Oliv.) Diels (Apiaceae; Angelica radix and rhizome), *Paeonia lactiflora* Pall. (Paeoniaceae; Paeonia root), *Mentha canadensis* L. (Lamiaceae; Mentha aerial part), *Ephedra sinica* Stapf (Ephedraceae; Ephedra root and rhizome), *Forsythia suspensa* (Thunb.) Vahl (Oleaceae; Forsythia fruit), Crystallized sodium sulfate, *Scutellaria baicalensis* Georgi (Lamiaceae; Scutellaria root), *Platycodon grandiflorus* (Jacq.) A.DC. (Campanulaceae; Platycodon root), Talc, *Glycyrrhiza glabra* L. (Fabaceae; Glycyrrhiza radix et rhizome), *Nepeta tenuifolia* Benth. (Lamiaceae; Nepeta aerial part), *Atractylodes macrocephala* Koidz. (Asteraceae; Atractylodes rhizome)	Shaking palsy

(Continued on following page)

TABLE 2 | (*Continued*) Classic traditional Chinese medicines with potential benefits for treating Parkinson's disease–associated symptoms.

Period	Text	Prescription	Decoction composition	Symptom(s)
Ming dynasty (1368–1644 AD)	Zhengzhi Zhunsheng	Jin Ya wine	Jinya Shi, *Bassia scoparia* (L.) A.J.Scott (Amaranthaceae; Bassia fruit), *Rehmannia glutinosa* (Gaertn.) DC. (Orobanchaceae; Rehmannia rhizome and root), *Lepyrodiclis holosteoides* (C. A. Mey.) Fisch. et Mey. (Caryophyllaceae; Lepyrodiclis root), *Aconitum carmichaeli* Debeaux (Ranunculaceae; Aconitum root tuber), *Saposhnikovia divaricata* (Turcz. ex Ledeb.) Schischk. (Apiaceae; Saposhnikovia rhizome), *Asarum sieboldii* Miq. (Aristolochiaceae; Asarum radix and rhizome), *Lllicium Lanceolatum* A. C. Smith (Winteraceae; Lllicium leaf), *Zanthoxylum bungeanum* Maxim. (Rutaceae; Zanthoxylum fruit), *Hansenia weberbaueriana* (Fedde ex H.Wolff) Pimenov and Kljuykov (Apiaceae; Hansenia radix et rhizome)	DystoniaSpasm and difficulty walking
		Ding Zhen Wan	*Gentiana macrophylla* Pall. (Gentianaceae; Gentiana root), *Saposhnikovia divaricata* (Turcz. ex Ledeb.) Schischk. (Apiaceae; Saposhnikovia rhizome), *Nepeta tenuifolia* Benth. (Lamiaceae; Nepeta aerial part), *Achyranthes bidentata* Blume (Amaranthaceae; Achyranthes radix), *Buthus martensii* Karsch(Scorpio; Buthus dried), *Gastrodia elata* Blume (Orchidaceae; Gastrodia rhizome), *Asarum sieboldii* Miq. (Aristolochiaceae; Asarum radix and rhizome), *Conioselinum anthriscoides* 'Chuanxiong' (Apiaceae; Conioselinum rhizome), *Paeonia lactiflora* Pall. (Paeoniaceae; Paeonia root), *Angelica sinensis* (Oliv.) Diels (Apiaceae; Angelica radix and rhizome), *Rehmannia glutinosa* (Gaertn.) DC. (Orobanchaceae; Rehmannia rhizome and root), *Astragalus mongholicus* Bunge (Fabaceae; Astragalus radix), *Atractylodes macrocephala* Koidz. (Asteraceae; Atractylodes rhizome)	
	Qixiao Liang Fang	Da Huoluo Dan	*Dienagkistrodon acutus* (Viperidae; Dienagkistrodon dried), *Zaocys dhumnades* Cantor (Colubridae; Zaocys dried), *Ephedra sinica* Stapf (Ephedraceae; Ephedra root and rhizome), *Asarum sieboldii* Miq. (Aristolochiaceae; Asarum radix and rhizome), *Buthus martensii* Karsch(Scorpio; Buthus dried), *Anemone raddeana* Regel (Ranunculaceae; Anemone leaf), *Paeonia lactiflora* Pall. (Paeoniaceae; Paeonia root), *Cyrtomium fortunei* J.Sm. (Polypodiaceae; Cyrtomium whole plant), *Saposhnikovia divaricata* (Turcz. ex Ledeb.) Schischk. (Apiaceae; Saposhnikovia rhizome), *Pueraria montana* var. lobata (Willd.) Maesen and S.M.Almeida ex Sanjappa and Predeep (Fabaceae; Pueraria radix et root), *Commiphora myrrha* (T.Nees) Engl. (Burseraceae; Commiphora oleo gum resin), *Boswellia carteri* Birdw. (Burseraceae; Boswellia resin exuding from the bark), *Calamus draco* Willd. (Arecaceae; Calamus fruit), Cinnabaris, Rhinoceros (Rhinocerotidae; Rhinoceros cornu), *Pheretima pectinifera* Michaelsen(Lumbricus; Pheretima drried), *Glycyrrhiza glabra* L. (Fabaceae; Glycyrrhiza radix and rhizome), *Syzygium aromaticum* (L.) Merr. and L.M.Perry (Myrtaceae; Syzygium flower), silkworm larva, moschus, *Dryobalanops aromatica* C.F.Gaertn. (Dipterocarpaceae; Dryobalanops resin), *Cinnamomum subavenium* Miq. (Lauraceae; Cinnamomum bark), *Alpinia hainanensis* K.Schum. (Zingiberaceae; Alpinia seed), *Hansenia weberbaueriana* (Fedde ex H.Wolff) Pimenov and Kljuykov (Apiaceae; Hansenia radix et rhizome), Tiger bone, *Scrophularia ningpoensis* Hemsl. (Scrophulariaceae; Scrophularia root), *Bos taurus domesticus* (Bovidae; cattle calculus), *Achyranthes bidentata* Blume (Amaranthaceae; Achyranthes radix), *Gastrodia elata* Blume (Orchidaceae; Gastrodia rhizome), *Agastache rugosa* (Fisch. and C.A.Mey.) Kuntze (Lamiaceae; Agastache leaf), *Bambusa textilis* McClure (Poaceae; Bambusa dried mass of secretion), *Chinemys reevesii* (Gray) (Testudinidae; Chinemys carapace), *Panax ginseng* C.A.Mey. (Araliaceae; Panax radix et rhizome), *Reynoutria multiflora* (Thunb.) Moldenke (Polygonaceae; Reynoutria radix), *Angelica dahurica* (Hoffm.) Benth. and Hook.f. ex Franch. and Sav. (Apiaceae; Angelica root), *Lindera aggregata* (Sims) Kosterm (Lauraceae; Lindera aggregata rhizome), *Styrax tonkinensis* (Pierre) Craib ex Hart. (Dtyracaceae; Styrax resin), *Citrus* × *aurantium* L. (Rutaceae; Citrus fruit), *Cyperus rotundus* L. (Cyperaceae; Cyperus rhizome and radix), *Wurfbainia vera* (Blackw.) Skornick. and A.D.Poulsen (Zingiberaceae; Wurfbainia fruit), *Davallia trichomanoides* Blume (Polypodiaceae; Davallia rhizome), *Coptis chinensis* Franch. (Ranunculaceae; Coptis rhizome), *Smilax glabra* Roxb. (Smilacaceae; Smilax rhizome), *Scutellaria baicalensis* Georgi (Lamiaceae; Scutellaria root), *Atractylodes macrocephala* Koidz. (Asteraceae; Atractylodes rhizome), *Rehmannia glutinosa* (Gaertn.) DC. (Orobanchaceae; Rehmannia rhizome and root), *Pinus massoniana* Lamb. (Pinaceae; Pinus essential oil), *Rheum officinale* Baill. (Polygonaceae; Rheum radix et rhizome), *Angelica sinensis* (Oliv.) Diels (Apiaceae; Angelica radix and rhizome), *Aucklandia costus* Falc. (Asteraceae; Aucklandia radix), *Aquilaria sinensis* (Lour.) Spreng. (Thymelaeaceae; Aquilaria resin containing wood)	Spasm and difficulty walking

patients' non-motor symptoms, depression, and anxiety (Li et al., 2012; Wang and Zhang, 2013; Guan et al., 2016). Acupuncture has a strong clinical effect on the motor symptoms of PD, such as tremor and muscle stiffness, as well as non-motor symptoms, such as disorders of mood and cognition and autonomic dysfunction (Noh et al., 2017).

TCM prescriptions have significant clinical effects in the treatment of PD. The differentiation of syndromes and treatment starts from the pathogenesis of PD and focuses on replenishing the liver and kidney and combining treatments, such as promoting blood circulation to remove blood stasis, extinguishing wind, relieving spasm, and resolving phlegm and detoxification, which can all relieve the motor symptoms of PD and protect the dopaminergic neurons (Wang et al., 2018; Cai et al., 2019; Zhang et al., 2019; Luan et al., 2020). Studies have found that TCM prescriptions and compounds used for the treatment of PD have antioxidant effects, which can affect mitochondrial function and intracellular antioxidant activity, regulate dopamine metabolism and iron metabolism, and protect neurons.

Pueraria montana var. lobata (Willd.) Maesen and S.M.Almeida ex Sanjappa and Predeep (Fabaceae; Pueraria radix) can relieve stiff muscles and promote the curative effect on tremor syndrome. Shanghan Zabing Lun proposed the use of Gegen Tang in treating the tension spots in the nape. Several studies have proven that the Guizhi jia Gegen decoction, which comprises *Pueraria montana* var. lobata (Willd.) Maesen and S.M.Almeida ex Sanjappa and Predeep (Fabaceae; Pueraria radix), is beneficial in the treatment of PD and could improve the movement of patients, ameliorate pain, have a good effect on PD patients with sleep disorders, reduce the dosage levels of L-dopamine, and improve the quality of life particularly of advanced patients (Zhu, 2002; Lian and Luo, 2008; Zheng et al., 2018). Its manifold actives intervene in neurological diseases. Puerarin is one of the major medicinal effective ingredients in *Pueraria montana* var. lobata (Willd.) Maesen and S.M.Almeida ex Sanjappa and Predeep (Fabaceae; Pueraria radix). Puerarin may protect dopaminergic neurons in various neurotoxin models of PD (Li et al., 2003; Zhang et al., 2014). It has been found to effectively ameliorate the MPTP-induced motor abnormalities in MPTP-lesioned mice and protect primary rat midbrain neurons against MPP+-induced toxicity *via* progesterone receptor signaling. Progesterone receptor modulates neuroprotective and regenerative responses in Parkinson's disease and related neurological diseases (Zhao et al., 2020). Another active is *Isodon lophanthoides* var. gerardianus, which lowers the risk of cerebrovascular disease, improves cerebral circulation, and induces the impairment of the neurons (Wang et al., 2008; Lai and Li, 2018).

TCM INTERVENES IN FERROPTOSIS

Chinese medicine is a treasure house of precious natural compounds, and many Chinese medicines have antioxidant effects. Moreover, studies have found that some TCMs can regulate ferroptosis (see **Table 3**), and the compound preparation of Naotai Fang can improve the cognitive functioning of rats with vascular cognitive dysfunction by regulating ferroptosis (Liao et al., 2015; Zeng et al., 2020). On the basis of the network pharmacological analysis of the ferroptotic cell death–associated targets of TCMs and compound preparations, it was found that quercetin, epigallocatechin-3-gallate, apigenin, luteolin, capsaicin, and genistein can act on multiple ferroptosis-associated target proteins. *Ginkgo biloba* L. (Ginkgoaceae; Ginkgo hojas desecadas), *Eriobotrya japonica* (Thunb.) Lindl. (Rosaceae; Eriobotrya leaves), *Phyllanthus emblica* L. (Phyllanthaceae; Phyllanthus dried ripe fruit), *Pueraria montana* var. lobata (Willd.) Maesen and S.M.Almeida ex Sanjappa and Predeep (Fabaceae; Pueraria radix et root), *Styphnolobium japonicum* (L.) Schott (Fabaceae; Styphnolobium flower bud or flower), *Ephedra sinica* Stapf (Ephedraceae; Ephedra root and rhizome), *Ligustrum lucidum* W.T.Aiton (Oleaceae; Ligustrum ripe fruit), and *Hippophae rhamnoides* L. (Elaeagnaceae; Hippophae ripe fruit) contain more targets that may have greater regulatory effects on the mechanisms of ferroptosis (Ou et al., 2019). Specifically, quercetin has been proven to upregulate the expression of hepcidin, hinder the absorption of intestinal iron, reduce the serum levels of iron, and reduce the bioavailability of iron. It is also an effective natural iron chelator and can reduce tissue damage caused by iron overload by maintaining iron levels in a steady state. Quercetin also removes ROS and other oxidizing substances and is a natural inhibitor of ferroptotic cell death (Sangkhae and Nemeth, 2017; Guo et al., 2018; Mazhar et al., 2018; Xiao et al., 2018). Baicalein, which is another natural ferroptosis inhibitor, can protect against damage due to oxidative stress. In comparison with classic inhibitors, such as liproxstatin-1, ferrostatin-1, β-mercaptoethanol, and deferoxamine mesylate, baicalein has more significant activity against ferroptotic cell death (Xie et al., 2016; Jing et al., 2018). Moreover, artemisinin and piperlongumine are inducers of ferroptotic cell death, and research on their antitumor aspects is increasing. They induce ferroptosis by increasing the ROS levels in tumor cells, reducing GSH levels, interfering with iron metabolism, and increasing Fe^{2+} concentrations (Eling et al., 2015; Greenshields et al., 2016). In comparison with the classic ferroptosis inducers, the TCMs and their active ingredients that have been reported to have a regulatory effect on ferroptosis have a greater number of regulatory targets and stable structures. However, further research is needed to sufficiently support the evidence provided by current studies.

Moxibustion at the Baihui and Sishencong acupoints in rats in the 6-hydroxydopamine model can increase the expression of the ferroptosis-associated proteins GPx4 and ferritin heavy chain 1 in the substantia nigra while increasing the activity of tyrosine hydroxylase to protect neuronal cells, and the mechanism may be related to the regulation of ferroptosis (Lu et al., 2019). Nevertheless, research on the use of TCM is limited, in particular, Chinese medicines and compound preparations, to treat PD through pathways associated with ferroptosis.

In the process of consulting the literature, we were surprised to have found that Puerarin, the main effective chemical component in the TCM *Pueraria montana* var. lobata (Willd.) Maesen and S.M.Almeida ex Sanjappa and Predeep (Fabaceae; Pueraria radix), is a natural ferroptosis inhibitor and can protect the neurons *via* the inhibited production of ROS. A high concentration of intracellular Ca^{2+} results in the differentiation of Y-79 cells to produce cytotoxic injury (Wang et al., 2016). It can reduce ROS levels, regulate iron homeostasis, and inhibit iron overload, thereby inhibiting ferroptosis (Roh et al., 2014; Chen et al., 2015). Alzheimer's disease is one of the common neurodegenerative brain disorders. Puerarin has been shown

TABLE 3 | Traditional Chinese medicines that regulate diseases *via* ferroptosis.

Traditional Chinese medicine		Category	Target tissue	Pathway	References
Active ingredient	Baicalein	Inhibitor	Pancreatic cancer cells	GPx4/Fenton reaction	Xie et al. (2016); Jing et al. (2018)
	Artemisinin	Derivant	Tumor cells	Oxidation pathway	Eling et al. (2015)
	Puerarin	Inhibitor	Cardiomyocytes	Oxidation pathway	Roh et al. (2014); Chen et al. (2015)
	Piperlongumine	Derivant	Tumor cells	Oxidation pathway	Greenshields et al. (2016)
	Quercetin	Inhibitor	Intestinal cells	Fenton reaction	Sangkhae and Nemeth (2017); Guo et al. (2018); Mazhar et al. (2018); Xiao et al. (2018)
Compound preparation	Naotai Fang	Inhibitor	Neurons	GPx4/oxidation pathway	Liao et al. (2015); Zeng et al. (2020)
	Huangqin Tang	Inhibitor	Colon cells	GPx4	Wu et al. (2021)
Non-medicinal	Moxibustion	Inhibitor	Neurons	GPx4	Lu et al. (2019)

to suppress iron overload in the cerebral cortex and improve spatial learning and memory disorders in mice with Alzheimer's disease, although the underlying mechanism remains unclear (Zhang et al., 2018). TCM preparations have strong clinical effects on both motor symptoms and non-motor symptoms of PD, and their mechanisms of action mainly focus on antioxidant activity, dopamine metabolism, iron metabolism, and so on. However, research on the use of TCM in the treatment of PD through pathways associated with ferroptosis is limited.

CONCLUSION

Ferroptosis is closely associated with various neurodegenerative diseases, and the ferroptosis of dopaminergic neurons is an important recently discovered pathogenetic mechanism of PD. The use of an iron chelator is the latest method for the treatment of PD. Slowing the progression of PD may be achieved by blocking iron-dependent death pathways, for example, upregulating or activating GSH to enable cell repair or inhibiting iron-dependent proline hydroxylase. TCM has a long history in the treatment of PD and has significant effects in the early prevention and clinical treatment of PD. However, research on the use of TCM in the treatment of PD through pathways associated with ferroptosis is limited. Moreover, TCM preparations have strong regulatory effects on ferroptosis to reverse the progress of the disease, which is useful in the development of the treatment methods for PD.

Some evidence on the use of TCM in the treatment of PD through pathways associated with ferroptosis may be insufficient because of the lack of systematic reviews. Moreover, at present, data on the relationship among TCM, PD, and ferroptosis are limited. However, more evidence has proven that TCM can treat other diseases by intervening in ferroptosis, such as tumors, angiocardiopathy, and cerebrovascular and other neurological diseases. These indirect pieces of evidence suggest that ferroptosis may be a valuable pathway in the research of Chinese herbal medicine intervention in PD.

Pueraria montana var. lobata (Willd.) Maesen and S.M.Almeida ex Sanjappa and Predeep (Fabaceae; Pueraria radix) has been prescribed for the treatment of PD. Clinical studies showed that the Guizhi jia Gegen decoction was based on *Pueraria montana* var. lobata (Willd.) Maesen and S.M.Almeida ex Sanjappa and Predeep (Fabaceae; Pueraria radix) containing medicine ingredients together which had effectiveness on patients with PD. The active formulations of *Pueraria montana* var. lobata (Willd.) Maesen and S.M.Almeida ex Sanjappa and Predeep (Fabaceae; Pueraria radix) could inhibit the death of nerve cells through the inhibition of enzymatic activities being related to the key genetic PD, and it could induce neuron apoptosis. Puerarin, which is a natural ferroptosis inhibitor, can protect against damage due to oxidative stress and restrain ferroptotic cell death. Moreover, *Pueraria montana* var. lobata (Willd.) Maesen and S.M.Almeida ex Sanjappa and Predeep (Fabaceae; Pueraria radix) and its compounds exhibit a protective effect on nerve cells if they intervene in PD by regulating neuronal ferroptosis. Hence, our future studies will be focused on the development of the treatment methods for PD.

AUTHOR CONTRIBUTIONS

JL and ZC conceived the idea. LW and ML prepared the manuscript. TM and DY reviewed the draft. NL, JC, YZ, and YH provided important information for the completion of this manuscript.

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Synergic Neuroprotection Between *Ligusticum Chuanxiong* Hort and Borneol Against Ischemic Stroke by Neurogenesis *via* Modulating Reactive Astrogliosis and Maintaining the Blood–Brain Barrier

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Background: *Ligusticum chuanxiong* Hort (LCH) is a famous ethnomedicine in Asia known for its excellent output on stroke treatment, and borneol usually acts as an assistant for its reducing permeability of the blood–brain barrier (BBB) after stroke. Although their synergy against brain ischemia was verified in previous studies, the potential mechanism is still unknown.

Methods: The research aimed to explore the exact synergic mechanisms between LCH and borneol on neurogenesis within the areas of the dentate gyrus and subventricular zone. After treating middle cerebral artery occlusion rats with LCH (0.1 g/kg) and/or borneol (0.08 g/kg), the neurological severity score, brain infarct ratio, Nissl staining, Evans blue permeability, BBB ultrastructure, and expressions of von Willebrand factor and tight junction–associated proteins were measured. Co-localizations of $Nestin^+/BrdU^+$ and $doublecortin^+/BrdU^+$, and expressions of neuronal nuclei (NeuN) and glial fibrillary acidic protein (GFAP) were observed under a fluorescence microscope. Moreover, astrocyte polarization markers of complement component 3 and pentraxin 3, and relevant neurotrophins were also detected by immunoblotting.

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Abbreviations: ACs: astrocytes, BBB: blood–brain barrier, bFGF: basic fibroblast growth factor, BMECs: brain microvascular endothelial cells, BO: borneol, BSA: bovine serum albumin, C3: complement component 3, CNS: central nervous system, CNTF: ciliary neurotrophic factor, DAPI: 4′,6–diamidino–2–phenylindole, DCX: doublecortin, DG: dentate gyrus, GAPDH: glyceraldehyde-3–phosphate dehydrogenase, GC–MS: gas chromatography–mass spectroscopy, GFAP: glial fibrillary acidic protein, JAM–3: junctional adhesion molecule 3, LCH: *Ligusticum chuanxiong* Hort, MCAO: middle cerebral artery occlusion, NeuN: neuronal nuclei, NGF: nerve growth factor, NSCs: neural stem cells, NSS: neurological severity scores, PTX3: pentraxin 3, PVDF: polyvinylidene fluoride, RIPA: radio-immunoprecipitation assay, rtPA: recombinant tissue plasminogen activator, SDS–PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis, SVZ: subventricular zone, TBST: tris-buffered saline Tween, TJs: tight junctions, TN–3: neurotrophin-3, TrkB: tyrosine kinase receptor B, TTC: 2,3,5–triphenyltetrazolium chloride, UPLC–MS/MS: ultra-performance liquid chromatography–tandem mass spectrometry, VEGF: vascular endothelial growth factor, VEGFR2: vascular endothelial growth factor receptor 2, vWF: von Willebrand factor, ZO–1: Zonula occludens–1.

Results: Basically, LCH and borneol had different focuses, although both of them decreased infarct areas, and increased quantity of Nissl bodies and expression of brain-derived neurotrophic factor. LCH increased the neurological severity score, $NeuN^+$ cells, and the ratios of $Nestin^+/BrdU^+$ and $doublecortin^+/BrdU^+$, and decreased $GFAP^+$ cells and ciliary neurotrophic factor expression. Additionally, it regulated the expressions of complement component 3 and pentraxin 3 to transform astrocyte phenotypes. Borneol improved BBB ultrastructure and increased the expressions of von Willebrand factor, tight junction–associated proteins, vascular endothelial growth factor, and vascular endothelial growth factor receptor 2. Unexpectedly, their combined therapy showed more obvious regulations on the Nissl score, Evans blue permeability, $doublecortin^+/BrdU^+$, $NeuN^+$ cells, brain-derived neurotrophic factor, and vascular endothelial growth factor than both of their monotherapies.

Conclusions: The results indicated that LCH and borneol were complementary to each other in attenuating brain ischemia by and large. LCH mainly promoted neural stem cell proliferation, neurogenesis, and mature neuron preservation, which was probably related to the transformation of reactive astrocytes from A1 subtype to A2, while borneol preferred to maintain the integrity of the BBB, which provided neurogenesis with a homeostatic environment.

Keywords: ***Ligusticum chuanxiong*** **Hort., borneol, ischemic stroke, neurogenesis, blood–brain barrier**

INTRODUCTION

Stroke, as a leading cause of death and disability, attacks about 15 million peoples worldwide each year, and ischemic stroke, caused by deficiency of cerebral blood flow, makes up about 80–85% of all strokes (Alwjwaj et al., 2021). Except antithrombotic therapy, there is no ideal therapy in clinic. However, the antithrombotic drugs, as represented by recombinant tissue plasminogen activator (rtPA), only provided a transient therapy window within 4.5 h (Lansberg et al., 2009), and even usually produces a wide range of side effects, such as bleeding, dizziness, and headache (Liu et al., 2018). So, discovering novel, highly effective, and low toxicity anti-ischemic stroke drugs with clear mechanism is one of the hot topics in pharmacologic research.

Neurogenesis, developed by endogenous neural stem cells (NSCs) with the ability to replace damaged neurons, is regarded as a potential therapeutic strategy of brain ischemia (Lindvall and Kokaia, 2011; Abe et al., 2012). Additionally, it has been demonstrated that NSCs are restricted within the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone of the dentate gyrus (DG) in the normal adult brains (Navarro Negredo et al., 2020; Ottoboni et al., 2020), and is activated by ischemic stress (Hou et al., 2017). Nestin, DCX, and GFAP are usually regarded as biomarkers of neural stem cells, newborn neurons, and astrocytes, respectively (Chen et al., 2020; Yin et al., 2020). Additionally, a growing number of reports suggest that an array of neurotrophic factors are synthesized and secreted into the neurogenic microenvironment to address ischemic stroke (Wu, 2005; Hedayatpour et al., 2018; Zalewska et al., 2020). The identified factors and receptors include brain-derived neurotrophic factor (BDNF), tyrosine kinase receptor B (TrkB) (Jiang et al., 2021), nerve growth factor (NGF) (Zalewska et al., 2020), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor 2 (VEGFR2) (Fei et al., 2018), ciliary neurotrophic factor (CNTF) (Jia et al., 2018), and neurotrophin-3 (NT-3) (Cruz et al., 2015). These neurotrophins produce different regulations on proliferation of NSCs, regeneration of neurons and astrocytes (ACs), and even maintenance of the blood–brain barrier (BBB) *via* a complex network in postischemic brain.

It is reported that there are two phenotypes of ACs, known as A1 and A2, in reactive astrocytosis. A2 ACs, marked with pentraxin 3 (PTX3), participate in the developments of neurons and synapses *via* releasing various neurotrophins, while A1 ACs, marked with complement component 3 (C3), is considered to be harmful because of its ability to kill central nervous system (CNS) neurons (Zamanian et al., 2012). Moreover, it is demonstrated that both long-term hypoperfusion and ischemia induce the increase of A1-type ACs, and even form a glial scar (Miyamoto et al., 2020). Thus, the transformation of AC phenotypes from A1 to A2 is regarded as a potential treatment strategy for ischemic stroke.

According to the theory of traditional Chinese Medicine, stroke is caused by blood stasis, and its treatment mainly depends on stasis-eliminating therapy. *Ligusticum chuanxiong* Hort (LCH) is one of the most common Chinese herbal medicines possessing stasis-eliminating function (Li et al., 2015; Chen et al., 2018). LCH injection is its commercial product and widely used for ischemic stroke patients in Asia. Previous studies suggest that LCH reduces cerebral infarct and inflammatory reaction, improves neurological behavior (Ip et al.,

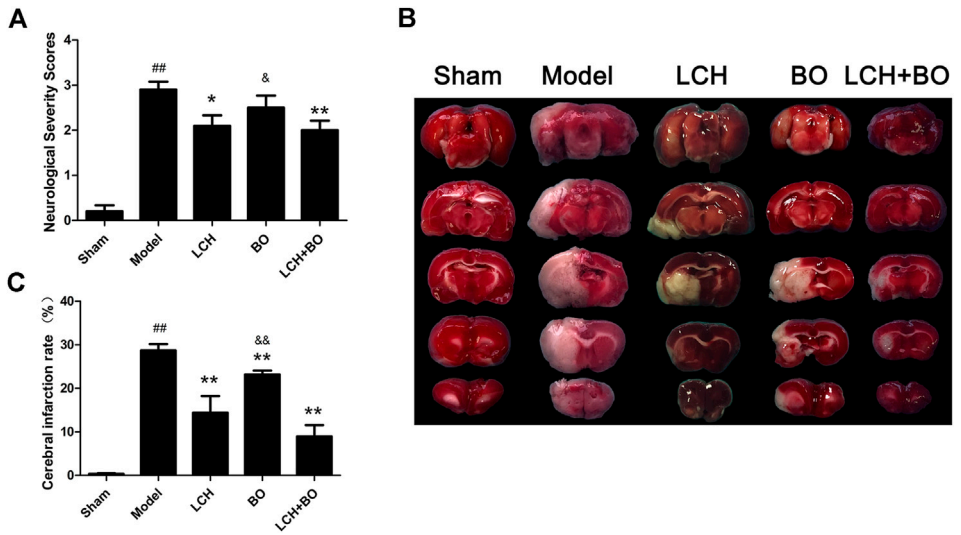

FIGURE 1 | Neurological severity scores and cerebral infarction rate of MCAO rats. **(A)** The results of neurological severity scores (n = 10). **(B)** Representative pictures of TTC staining. **(C)** The results of cerebral infarction ratio (n = 6). $^{\#\#}p < 0.01$ compared to the sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the model group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ compared to the LCH + BO group.

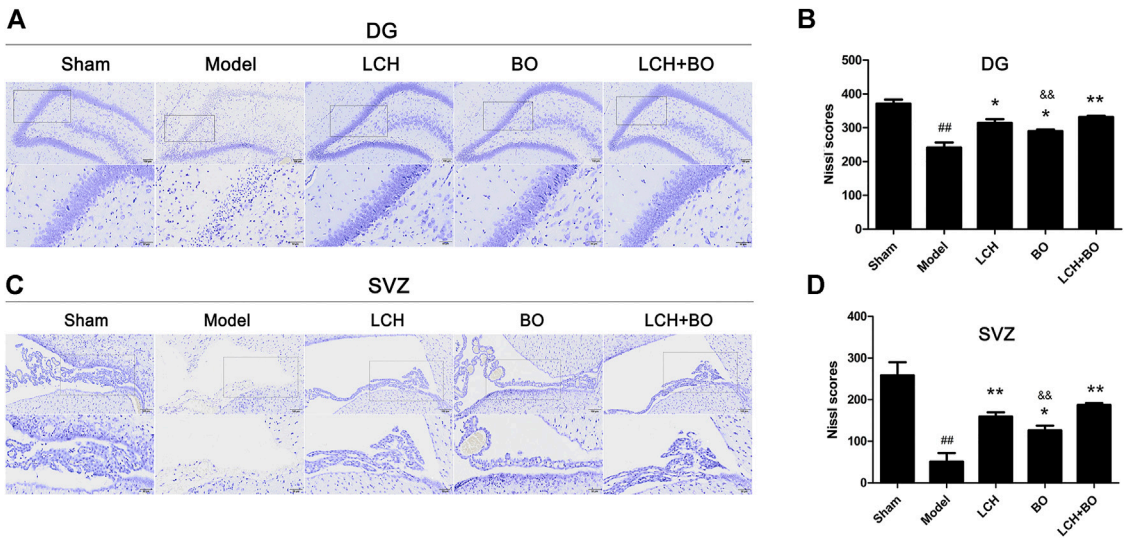

FIGURE 2 | Nissl scores within DG and SVZ of MCAO rats. **(A)** Representative pictures of Nissl staining in the DG area. **(B)** The results of Nissl scores in the DG area (n = 3). **(C)** Representative pictures of Nissl staining in the SVZ area. **(D)** The results of Nissl scores in the SVZ area (n = 3). $^{\#\#}p < 0.01$ compared to the sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the model group; $^{\&\&}p < 0.01$ compared to the LCH + BO group.

2016), and alleviates oxidative stress and neuronal apoptosis of middle cerebral artery occlusion (MCAO) rats (Gu et al., 2020). Borneol (BO), another Chinese ethnomedicine with a bicyclic terpene structure, is extracted from *Blumea balsamifera* (L.) DC. or *Cinnamomum camphora* (L.) Presl. There are numerous evidences indicating that BO significantly reduces brain water content, alleviates brain edema, and maintains the BBB in cerebral ischemic injury (Zhang X.-g. et al., 2017; Chen et al., 2019). Additionally, BO is more often used as an assistant in CNS treatment, especially for stroke, to produce a synergic therapeutic effect basing on traditional Chinese Medicine theory (Zhang X.-G. et al., 2017; Liao et al., 2020). Our early studies have confirmed that the combination of LCH and BO exerts a much better protection against cerebral ischemia than their monotherapies *via* downregulation of apoptosis, upregulation of autophagy, and angiogenesis (Yu et al., 2020a; Yu et al., 2020b). However, their

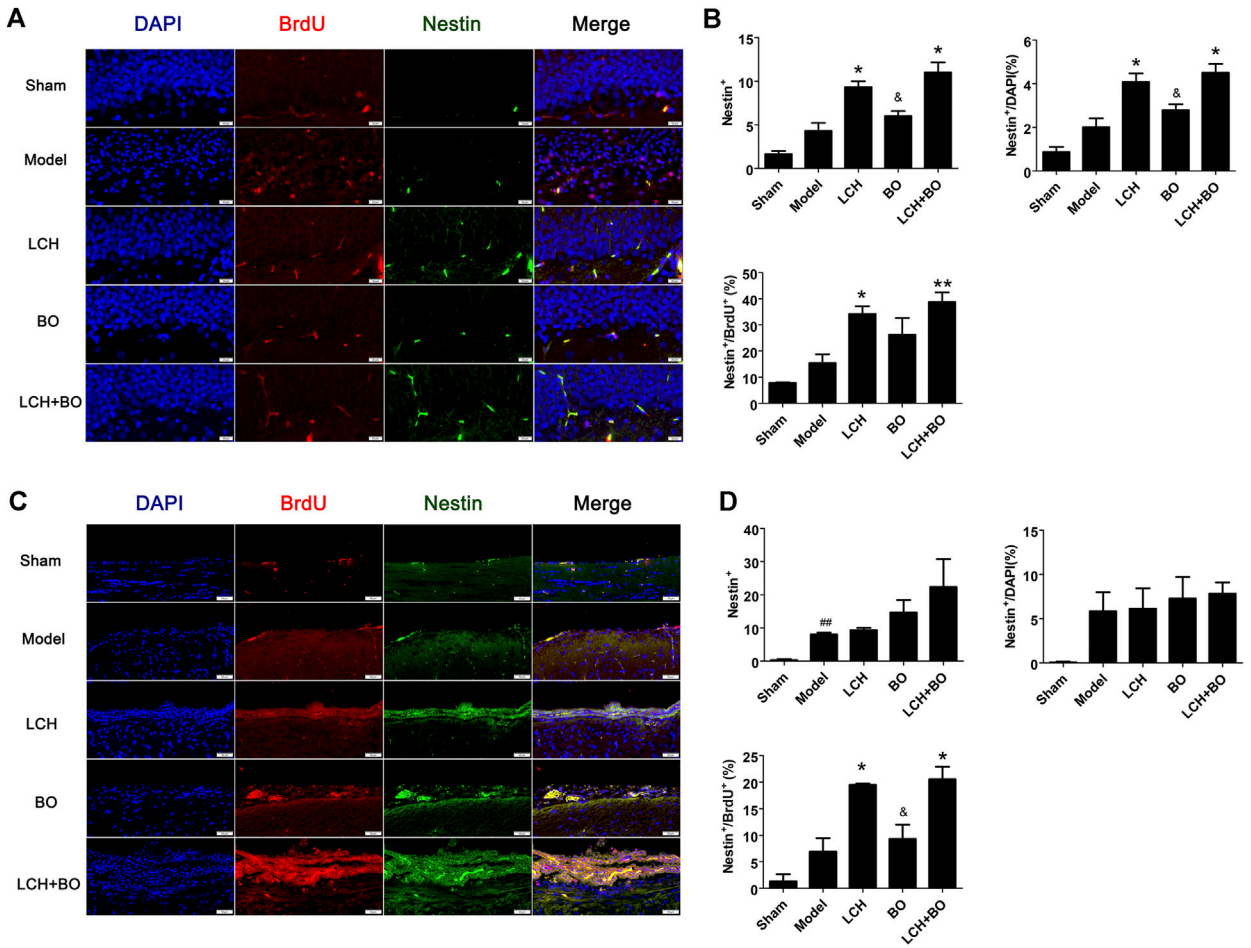

FIGURE 3 | NSC proliferation within DG and SVZ of MCAO rats. **(A)** Representative double-immunostaining images of Nestin$^+$/BrdU$^+$ in the DG area. **(B)** The results of Nestin$^+$ cells, Nestin$^+$/DAPI, and Nestin$^+$/BrdU$^+$ in the DG area ($n = 3$). **(C)** Representative double-immunostaining images of Nestin$^+$/BrdU$^+$ in the SVZ area. **(D)** The results of Nestin$^+$ cells, Nestin$^+$/DAPI, and Nestin$^+$/BrdU$^+$ in the SVZ area ($n = 3$). $^{\#\#}p < 0.01$ compared to the sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the model group; $^{\&}p < 0.05$ compared to the LCH + BO group.

respective focuses and potential synergic mechanism are still unclear.

In consideration of abovementioned results and the close relationship among neuron autophagy, neurogenesis, and NSC differentiation (Fleming and Rubinsztein, 2020), the present study was designed to explore the synergic mechanism between LCH and BO against stroke based on NSC differentiation, neurogenesis, and BBB maintenance.

MATERIALS AND METHODS

Materials

LCH injection, a steam distillation product of LCH, was provided by Changhai Hospital of Shanghai. A total of 59 compounds were identified by ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) technology, and 25 compounds were identified by the gas chromatography–mass spectroscopy (GC–MS) method. The detailed information is listed in **Supplementary File**. BO was purchased from Beijing Sanhe Pharmaceutical Co. Ltd., and its purity was 99.1%.

Animals

Healthy male SD rats (8 weeks, 300–350 g) were purchased from the Animal Center of Nanjing University of Chinese Medicine, maintained in a light/dark cycle (12 h/12 h) room, and freely accessible to food and water. The animal protocols of the study were approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine (No. 201901A007).

Procedure For Middle Cerebral Artery Occlusion

MCAO rat is a widely used animal model of ischemic stroke because it may produce similar pathological changes to stroke patients in clinic (Wu et al., 2020; Barahimi et al., 2021), and the procedure was similar to previous reports with minor

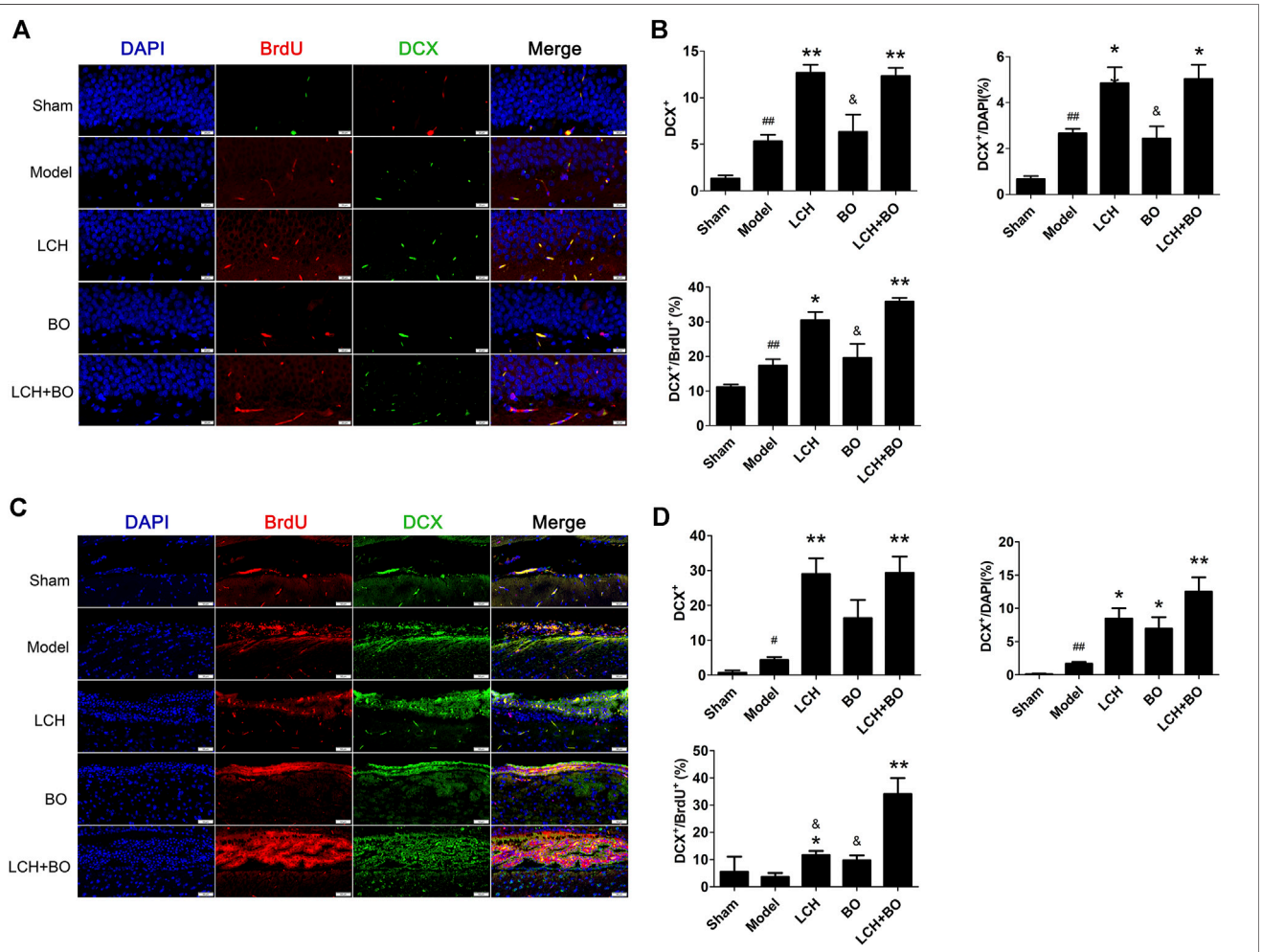

FIGURE 4 | Neurogenesis within DG and SVZ of MCAO rats. **(A)** Representative double-immunostaining images of $DCX^+/BrdU^+$ in the DG area. **(B)** The results of DCX^+ cells, DCX^+/DAPI, and $DCX^+/BrdU^+$ in the DG area ($n = 3$). **(C)** Representative double-immunostaining images of $DCX^+/BrdU^+$ in the SVZ area. **(D)** The results of DCX^+ cells, DCX^+/DAPI, and $DCX^+/BrdU^+$ in the SVZ area ($n = 3$). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to the sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the model group; $^{\&}p < 0.05$ compared to the LCH + BO group.

modifications (Costa et al., 2016; Ma et al., 2016). Briefly, the rat was anesthetized with 3% isoflurane in a chamber affiliated to a small animal anesthesia machine (RWD Life Science Co., LTD., China) and maintained with 1.5% isoflurane delivered through a face mask fitting the rat's head. After the left common carotid artery was exposed, the internal and external carotid arteries were separated from each other. Then the external carotid artery was ligated by an absorbable suture. A nylon thread (diameter 0.26–0.27 mm) with its top coated with silicone (length 6–7 mm and diameter 0.41–0.45 mm) was inserted into the internal carotid artery at a depth of 18–20 mm to block the middle cerebral artery, and reperfusion was followed 1 h later. The rats in the sham group underwent the same operation, except the insertion of nylon thread. The rat's body temperature was maintained at 37°C during the entire operation. 24 hours after the surgery, the rats with neurological severity scores (NSS) no less than three were used for the following study (Bieber et al., 2019).

Drug Treatment and BrdU Label

Rats were randomly divided into five groups, with 19 rats in each group: sham, model, LCH, BO, and LCH + BO groups. All rats were subjected to MCAO operation, except those in the sham group. The rats in the LCH, BO, and LCH + BO groups were treated with LCH injection (i.p. 0.1 g/kg) or/and BO (i.g. 0.08 g/kg). Both the sham and the model groups were given equal volume of PBS (i.p.) and liquid paraffin (i.g.). All drugs were given once daily for 7 days, including 4 days before the MCAO surgery and 3 days after that. Additionally, the rats used for immunofluorescence analysis were injected with 5-bromo-2'-deoxyuridine (BrdU, i.p. 50 mg/kg) once daily for the last 3 days.

Behavioral Test

After treatment for 7 days, the NSS were employed to evaluate neurological behaviors *via* a five-point Bederson scale (Bederson et al., 1986) (Bi et al., 2017). Increase of the score indicated decrease of neurological function. Specifically, 0: no abnormal

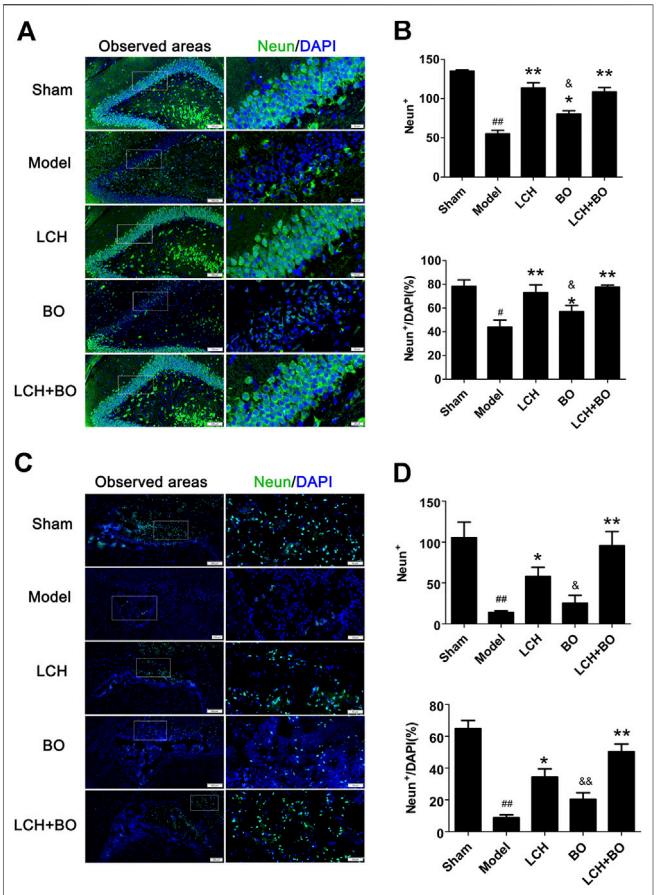

FIGURE 5 | Mature neuron preservation within DG and SVZ of MCAO rats. **(A)** Representative immunostaining images of NeuN in the DG area. **(B)** The results of $NeuN^+$ cells and $NeuN^+$/DAPI in the DG area ($n = 3$). **(C)** Representative immunostaining images of NeuN in the SVZ area. **(D)** The results of $NeuN^+$ cells and $NeuN^+$/DAPI in the SVZ area ($n = 3$). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to the sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the model group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ compared to the LCH + BO group.

behavior; 1: minor neurological deficiency (right forelimb bending when lifting its tail); 2: moderate neurological deficiency (decreased stability to left slight push) without cycling; 3: same neurological deficiency as grade 2, but cycling to right when moving; and 4: no spontaneous walking, or even unconsciousness.

Infarct Size Measurement

2,3,5-triphenyltetrazolium chloride (TTC) staining was performed to measure the ratio of infarct size. After being anesthetized with 2% isoflurane, the rat was sacrificed by decapitation, and then its brain was taken out gently. Five brain coronal slices with a thickness of 2 mm were made by a tissue microtome. Then the slices were immersed in a 0.1% TTC PBS solution at 37°C for 15 min. Both the infarct area and the total brain area were measured using ImageJ software, and the infarction ratio was the percentage of infarct area in the total brain area.

Score of Nissl Staining

The whole brain tissue was obtained and placed in a 4% paraformaldehyde PBS solution for 24 h. After dehydration, it was fixed in paraffin, and then cut into 4 μm coronal slices according to the stereotaxic coordinates of DG and SVZ (DG: AP −3.6 mm; SVZ: AP + 0.0 mm). Stained with 1% toluidine blue in accordance with the kit's instruction, the intact Nissl bodies in both DG and SVZ areas were quantified as Nissl scores using a microscope (IX71, Olympus, Tokyo, Japan) under five random fields.

Blood–Brain Barrier Permeability Evaluation

The rat was injected with 4 ml/kg of 2% Evans blue (EB) *via* caudal vein. 3 hours later, the rat was anesthetized with isoflurane and perfused with PBS *via* left ventricle to remove the intravascular EB. After pictures were taken, the content of EB in brain tissue was measured according to previous reports (Nozaki et al., 2018; Cheng et al., 2021). Briefly, the ischemic

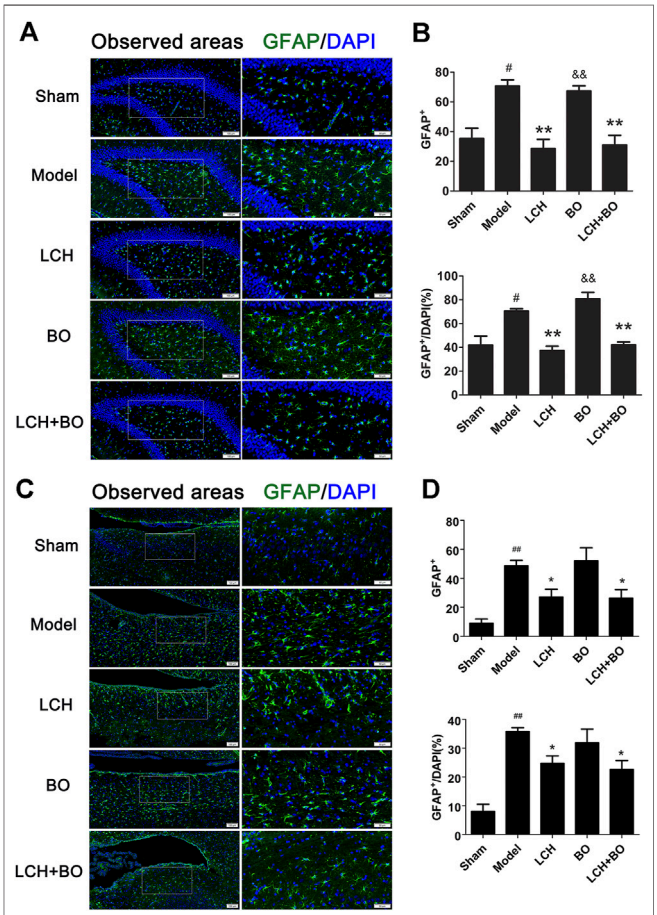

FIGURE 6 | ACs proliferation within DG and SVZ of MCAO rats. **(A)** Representative immunostaining images of GFAP in the DG area. **(B)** The results of $GFAP^+$ cells and $GFAP^+$/DAPI in the DG area ($n = 3$). **(C)** Representative immunostaining images of GFAP in the SVZ area. **(D)** The results of $GFAP^+$ cells and $GFAP^+$/DAPI in the SVZ area ($n = 3$). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to the sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the model group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ compared to the LCH + BO group.

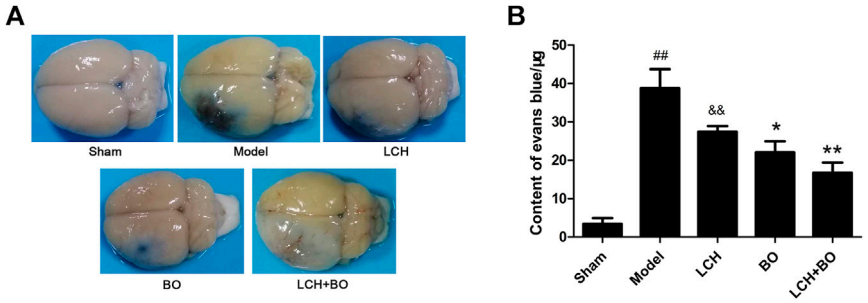

FIGURE 7 | BBB permeability evaluation of MCAO rats. **(A)** Representative brain pictures after the injection of Evans blue. **(B)** The contents of Evans blue in the brains of MCAO rats ($n = 6$). $^{\#\#}p < 0.01$ compared to the sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the model group; $^{\&\&}p < 0.01$ compared to the LCH + BO group.

side hemisphere was separated, weighted, and homogenized in 3 ml of formamide. Then the sample was incubated at 37°C for 48 h, followed by centrifugation at 10,000 g for 20 min. The OD value of the supernatant was measured by a microplate reader (BioTek Instruments, Vermont, United States) at the wavelength of 620 nm. The content of EB in the brain was calculated by an EB standard curve.

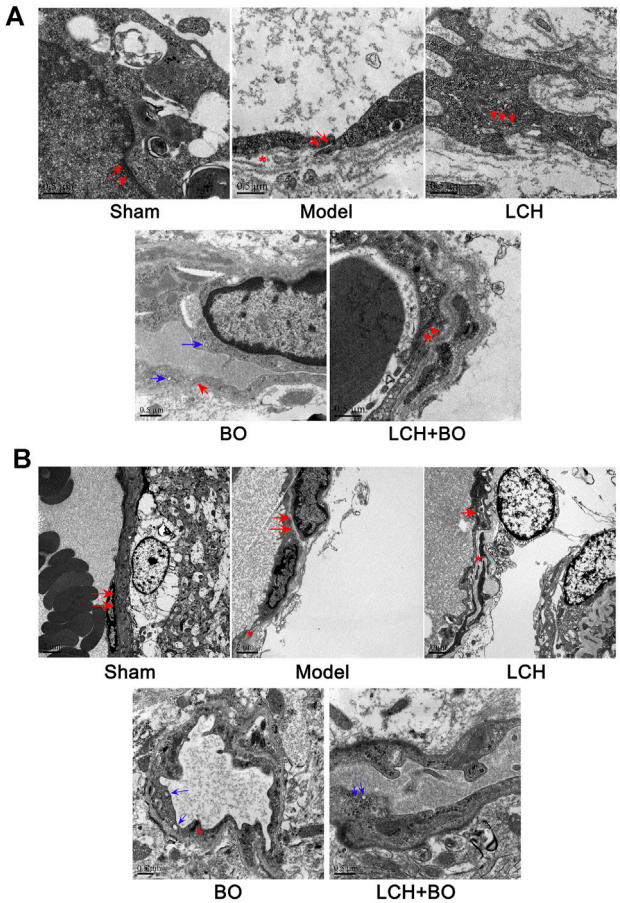

FIGURE 8 | BBB ultrastructures within DG and SVZ of MCAO rats. **(A)** Representative images of transmission electron microscope within the DG area. **(B)** Representative images of transmission electron microscope within the SVZ area. The red arrows denoted TJs. The blue arrows denoted pinocytotic vesicles. The red asterisks denoted cavities between the endothelium and basement membrane.

Ultrastructure Examination

The brain tissue was prepared as our previous report (Yu et al., 2013b). Briefly, a small piece of DG or SVZ tissue was removed carefully from rat brain, fixed in 2% glutaraldehyde for 4 h, and osmicated for 1 h at 4°C with 1% OsO_4 and 0.8% potassium ferricyanide. Then the brain section was dehydrated using acetone, embedded in Epon 812 epoxy resin, and prepared as an ultrathin section. Finally, the section was observed using a transmission electron microscope (H7650, Hitachi, Japan) after being stained with uranyl acetate and lead citrate.

Immunofluorescence Histochemistry

After being anesthetized with isoflurane and perfused with PBS, the rat brain was removed and fixed in 4% paraformaldehyde for 72 h, and then sliced into 4- to 8-μm-thick coronal pieces in accordance with the stereotaxic coordinates of DG and SVZ mentioned above. Subsequently, the slice was mounted on a glass substrate, rinsed with PBS three times, immersed in 10% donkey serum for 1 h, and incubated overnight at 4°C with corresponding rabbit primary antibody below. For detecting NSC proliferation and neurogenesis, the primary antibodies were BrdU (1:100, catalog number: ab6326, Abcam, Cambridge, MA, United States), Nestin (1:100, catalog number: ab221660, Abcam, Cambridge, MA, United States), and Doublecortin (DCX, 1:200, catalog number: ab18723, Abcam, Cambridge, MA, United States). For assessing the preservation of mature neurons and astrogliosis, the primary antibodies were neuronal nuclei (NeuN, 1:400, catalog number: ab177487, Abcam, Cambridge, MA, United States) and glial fibrillary acidic protein (GFAP, 1:400, catalog number: ab7260, Abcam, Cambridge, MA, United States). For evaluating the proliferation of brain microvascular endothelial cells (BMECs), the primary antibody was von Willebrand factor (vWF) (1:400, catalog number: ab6994, Abcam, Cambridge, MA, United States). For observing expressions of TJ-associated proteins on the BBB, the primary antibodies were claudin-5 (1:50, catalog number: 310,145, Sigma-Aldrich, Saint Louis, MO, United States), junctional adhesion molecule 3 (JAM-3, 1:100, catalog number: ab214194,

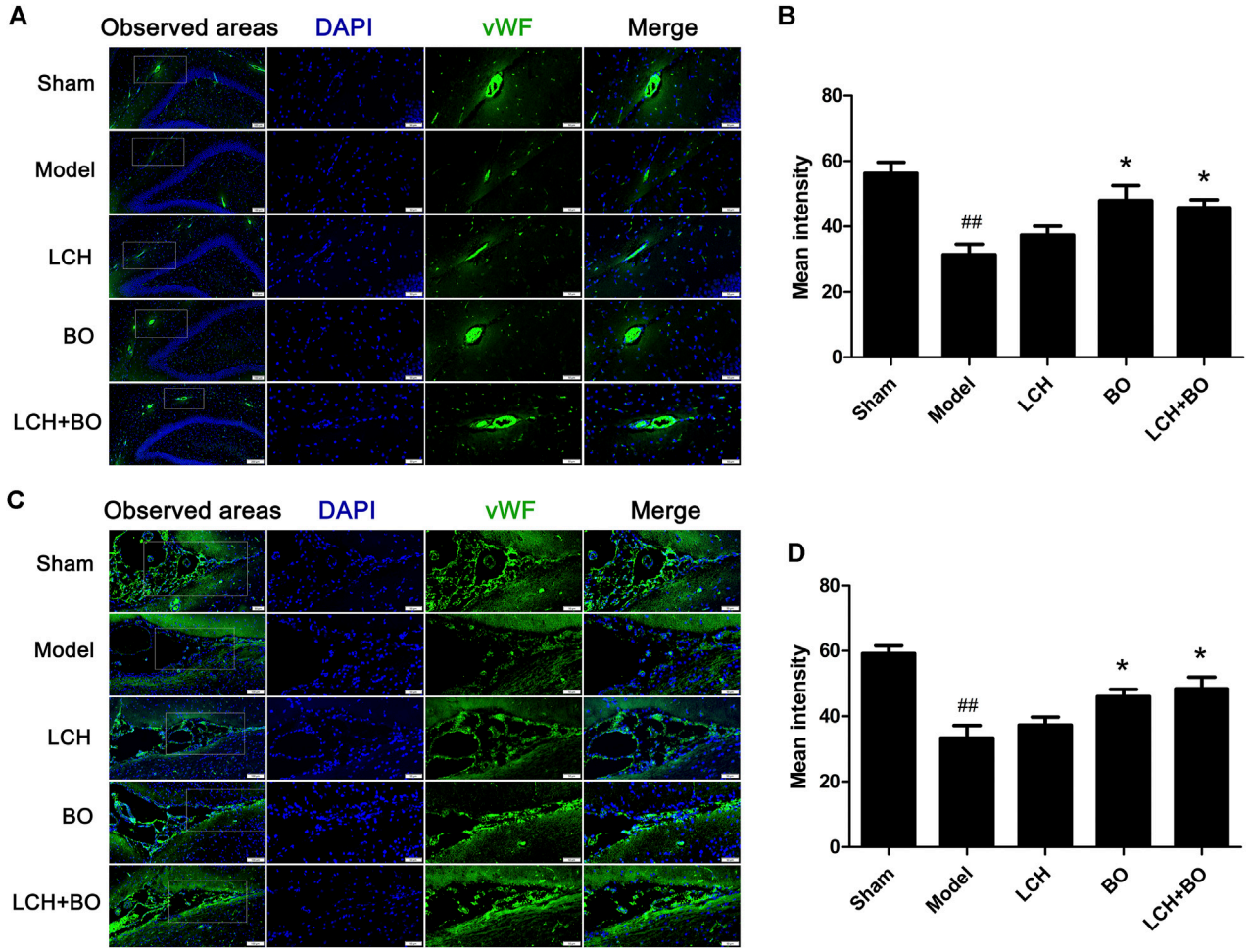

FIGURE 9 | Expressions of vWF within DG and SVZ of MCAO rats. **(A)** Representative immunostaining images of vWF in the DG area. **(B)** The results of average fluorescence intensities of vWF in the DG area (n = 3). **(C)** Representative immunostaining images of vWF in the SVZ area. **(D)** The results of average fluorescence intensities of vWF in the SVZ area (n = 3). $^{\#\#}p < 0.01$ compared to the sham group; $^{*}p < 0.05$ compared to the model group.

Abcam, Cambridge, MA, United States), occludin (1:50, catalog number: 71–1,500, Thermo Scientific, Waltham, MA, United States), and zonula occludens-1 (ZO-1, 1:100, catalog number: ab221547, Abcam, Cambridge, MA, United States). After the slice was incubated with second antibodies coupled with Alexa Fluor 488 (a green fluorescent dye) or Alexa Fluor 568 (a red fluorescent dye) for 1 h at room temperature, it was counterstained with 4′,6–diamidino–2–phenylindole (DAPI), and then visualized using a fluorescence microscopy (IX71, Olympus Tokyo, Japan). The fluorescence intensity was measured using ImageJ software.

Western Blot

DG and SVZ tissues were removed carefully, and the proteins were extracted using a radio-immunoprecipitation assay (RIPA) buffer (Jiangsu KeyGEN BioTECH Co., Ltd.). Proteins with equal content were separated on 4–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to 0.22 μm polyvinylidene fluoride (PVDF) membranes (Millipore). After being blocked by 5% bovine serum albumin (BSA), the membranes were incubated with the following primary antibodies overnight at 4°C: BDNF (1: 5,000, catalog number: ab108319, Abcam, Cambridge, MA, United States), bFGF (1:2000, catalog number: PA5–95284, Invitrogen, Carlsbad, CA, United States), CNTF (1:5,000, catalog number: 10,796–1–AP, Proteintech, Rosemont, IL, United States), NGF (1:1,000, catalog number: MA5–32067, Invitrogen, Carlsbad, CA, United States), NT-3 (1:2000, catalog number: 18,084–1–AP, Proteintech, Rosemont, IL, United States), TrkB (1:1,000, catalog number: 13,129–1–AP, Proteintech, Rosemont, IL, United States), VEGF (1:2000, catalog number: 19,003–1–AP, Proteintech, Rosemont, IL, United States), VEGFR2 (1:1,000, catalog number: ab39256, Abcam, Cambridge, MA, United States), C3 (1:2000, catalog number: ab200999, Abcam, Cambridge, MA, United States), PTX3 (1:1,000, catalog number: ab134920, Abcam, Cambridge, MA, United States), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:10,000, catalog number: 10,494–1–AP, Proteintech, Rosemont, IL, United States).

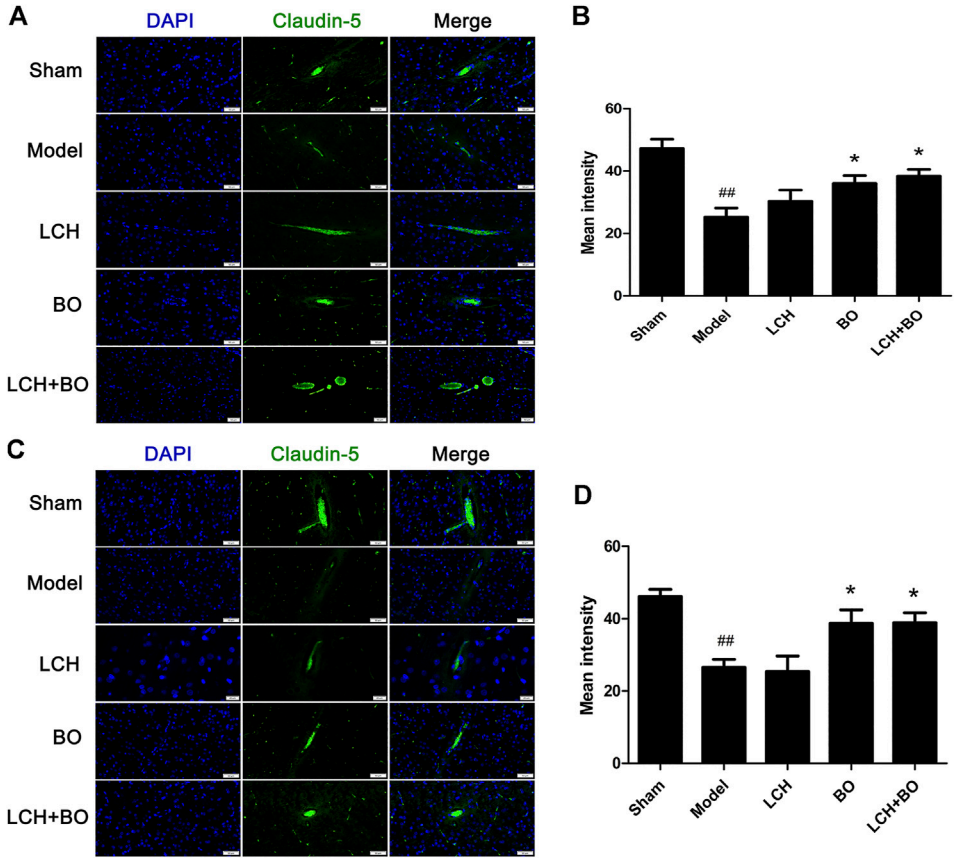

FIGURE 10 | Expressions of claudin-5 within DG and SVZ of MCAO rats. **(A)** Representative immunostaining images of claudin-5 in the DG area. **(B)** The results of average fluorescence intensities of claudin-5 in the DG area (n = 3). **(C)** Representative immunostaining images of claudin-5 in the SVZ area. **(D)** The results of average fluorescence intensities of claudin-5 in the SVZ area (n = 3). $^{\#\#}p < 0.01$ compared to the sham group; $^{*}p < 0.05$ compared to the model group.

After being washed by tris-buffered saline tween (TBST), the membranes were incubated with corresponding secondary antibodies for 1 h, and then washed three times. The chemiluminescence signals were detected by an ImageQuant LAS4000 mini (GE Healthcare Life Sciences, Piscataway, NJ, United States) and analyzed by ImageJ software.

Statistical Analysis

Data were presented as the mean ± SD and analyzed using a one-way analysis of variance (ANOVA). The Tukey test was used for multiple comparisons. GraphPad Prism 5.0 software was employed to perform statistical analysis, and $p < 0.05$ was considered statistically significant.

RESULTS

Ligusticum chuanxiong Hort Plays a Major Role in Improving Behavior Test in the Combination Therapy

As shown in **Figure 1A**, MCAO model rat displayed significant increase of NSS in comparison with the sham group ($p < 0.01$), which suggested that cerebral ischemia induced abnormal behavior and movement. With the single treatment of LCH, the NSS obviously reduced ($p < 0.05$), which indicated the recovery of neurological function. Although BO itself did not improve NSS, it elevated the therapeutic effect of LCH ($p < 0.01$). Additionally, the LCH + BO group exhibited a better performance than the BO group. These above results provided evidence for the synergy treatment of LCH and BO on cerebral ischemia, and confirmed the major role of LCH in behavior improvement during the combined therapy.

Ligusticum Chuanxiong Hort and Borneol Synergically Reduce Infarct Areas

TTC can dye normal brain tissue to red, while the ischemic region maintains white (**Figure 1B**). In the present study, LCH and BO markedly decreased infarct brain areas of MCAO rats ($p < 0.01$), which indicated that both of them ameliorated ischemia attack (**Figure 1C**). Interestingly, their combination exhibited a much more powerful protection than BO monotherapy ($p < 0.01$), which implied that the potential mechanisms of their protections might be different.

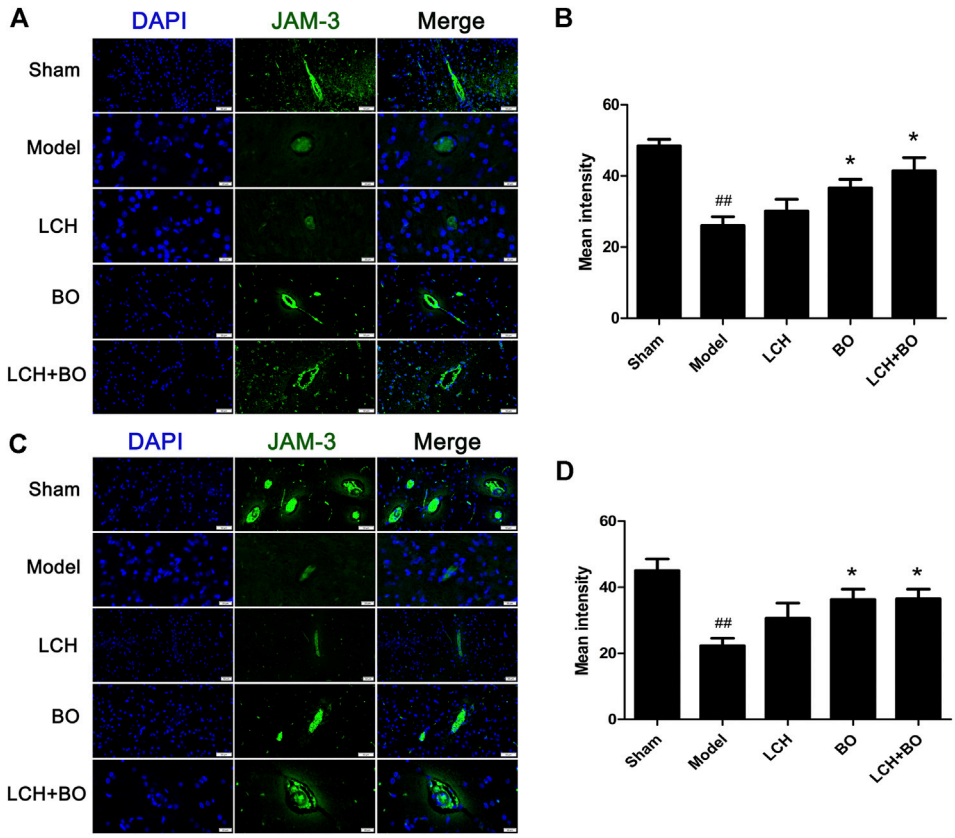

FIGURE 11 | Expressions of JAM-3 within DG and SVZ of MCAO rats. **(A)** Representative immunostaining images of JAM-3 in the DG area. **(B)** The results of average fluorescence intensities of JAM-3 in the DG area (n = 3). **(C)** Representative immunostaining images of JAM-3 in the SVZ area. **(D)** The results of average fluorescence intensities of JAM-3 in the SVZ area (n = 3). $^{\#\#}p < 0.01$ compared to the sham group; $^{*}p < 0.05$ compared to the model group.

Ligusticum chuanxiong Hort and Borneol Synergically Attenuate the Loss of Neurons in Dentate Gyrus and Subventricular Zone Regions

The morphological trait of Nissl bodies reflected the statue of neurons. Obviously, MCAO attack led to extensive death of neurons in both DG (**Figures 2A,B**) and SVZ regions (**Figures 2C,D**). Although both LCH and BO exhibited their improvements on Nissl scores ($p < 0.05$, 0.01), their degrees of protection were different. LCH displayed better maintenances on shape and amount of Nissl bodies than BO in both of the regions. Moreover, the score of the LCH + BO group was much more than that of the BO group ($p < 0.01$). Apparently, LCH played a major role in neuron maintenance during the combined therapy.

Ligusticum chuanxiong Hort Displays Significant Advantages on Neural Stem Cell Proliferation and Neurogenesis

The co-localizations of $Nestin^+/BrdU^+$ and $DCX^+/BrdU^+$ were detected to explore the synergic mechanism between LCH and BO on NSC proliferation and neurogenesis in the present study. It was demonstrated that ischemic stroke increased $Nestin^+$ cells in SVZ (**Figures 3C,D**) and DCX^+/DAPI in both SVZ and DG (**Figure 4**) ($p < 0.01$). Apparently, the hypoxic condition induced proliferations of NSCs and neurogenesis, which was similar to those of previous reports (Lin et al., 2015; Huang et al., 2020).

LCH increased $Nestin^+$, $Nestin^+$/DAPI, and $Nestin^+/BrdU^+$ in DG, and $Nestin^+/BrdU^+$ in SVZ ($p < 0.05$), which implied that LCH promoted the proliferation of NSCs (**Figure 3**). Although BO showed no effect on above indexes, it further elevated the effect of LCH on $Nestin^+/BrdU^+$ in DG ($p < 0.01$). In the assessments of neurogenesis (**Figure 4**), LCH increased DCX^+ cells, DCX^+/DAPI, and $DCX^+/BrdU^+$ in the two regions, while BO only enhanced DCX^+/DAPI in SVZ ($p < 0.05$,0.01). Besides, their combined treatment showed more obvious improvements on $DCX^+/BrdU^+$ in both the areas. The results suggested that LCH played key roles in NSC proliferation and neurogenesis, while BO further improved the effects of LCH to some extent.

Ligusticum chuanxiong Hort Plays an Important Role in Neuron Survival and Astrocyte Proliferation

NeuN and GFAP are usually used as the markers of mature neurons and ACs, respectively. The present study found that ischemic stroke induced proliferations of ACs and loss of mature neurons. LCH

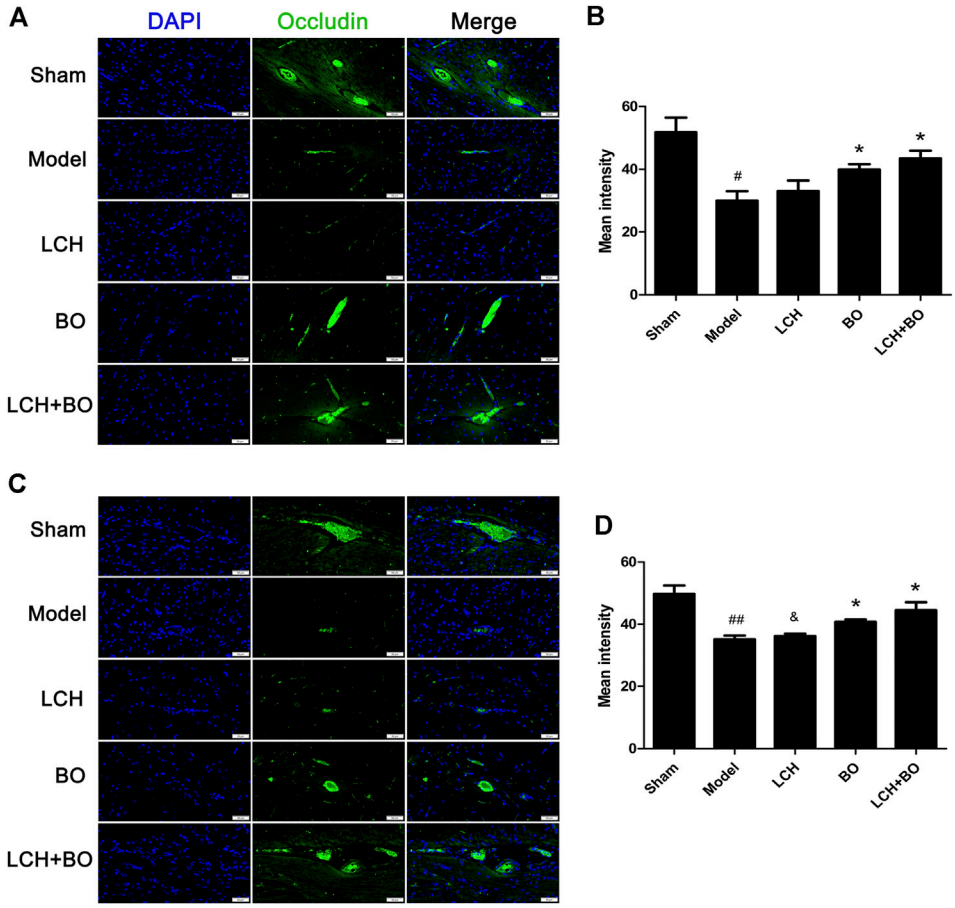

FIGURE 12 | Expressions of occludin within DG and SVZ of MCAO rats. **(A)** Representative immunostaining images of occludin in the DG area. **(B)** The results of average fluorescence intensities of occludin in the DG area (n = 3). **(C)** Representative immunostaining images of occludin in the SVZ area. **(D)** The results of average fluorescence intensities of occludin in the SVZ area (n = 3). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to the sham group; $^{*}p < 0.05$ compared to the model group; $^{\&}p < 0.05$ compared to the LCH + BO group.

increased $NeuN^+$ cells and $NeuN^+$/DAPI (**Figure 5**), and decreased $GFAP^+$ and $GFAP^+$/DAPI (**Figure 6**) in the two brain regions, while BO only enhanced $NeuN^+$ cells and $NeuN^+$/DAPI within the DG area ($p < 0.05$, 0.01). Besides, their combined treatment showed more increases on $NeuN^+$ cells and $NeuN^+$/DAPI in SVZ ($p < 0.01$). Similarly, the results suggested that LCH played main roles in preserving mature neurons and prohibiting AC proliferation, while BO strengthened the effect of LCH on neuron protection.

Borneol Plays a Key Role in Improving Blood–Brain Barrier Function in the Combined Therapy

EB, as a blue dye, is regarded as an indicator of BBB function (**Figure 7A**). The content of EB in the model rat brain was much more than that in the sham group (**Figure 7B**), which indicated that MCAO injury disrupted the BBB and enhanced its permeability. Compared to the model group, the LCH group showed no obvious effect on BBB function ($p > 0.05$), while BO markedly reduced the EB content in the brain tissue ($p < 0.05$). Moreover, their synergic therapy even showed a more obvious improvement on BBB function ($p < 0.01$). Apparently, unlike the above indexes on neurological function, BO, instead of LCH, played a key role in maintaining the BBB during the combined treatment.

Recovery of Blood–Brain Barrier Ultrastructure Owes to Borneol in the Combined Treatment

For exploring the potential BBB maintenance mechanism of BO, a transmission electron microscope technology was adopted to observe ultrastructures of the BBB. The results in DG (**Figure 8A**) and SVZ (**Figure 8B**) were similar on the whole. For BBB structures of the sham group, the cytomembrane boundaries of BMECs were clear and smooth without obvious pinocytosis. Particularly, the TJs between endothelial cells were normal without any gap. The connections between endothelium and basement membrane were also tight.

Yet, brain ischemia attacked BBB structures extensively and deeply. In the model group, cavities emerged between the endothelium and basement membrane, and the TJs were slightly

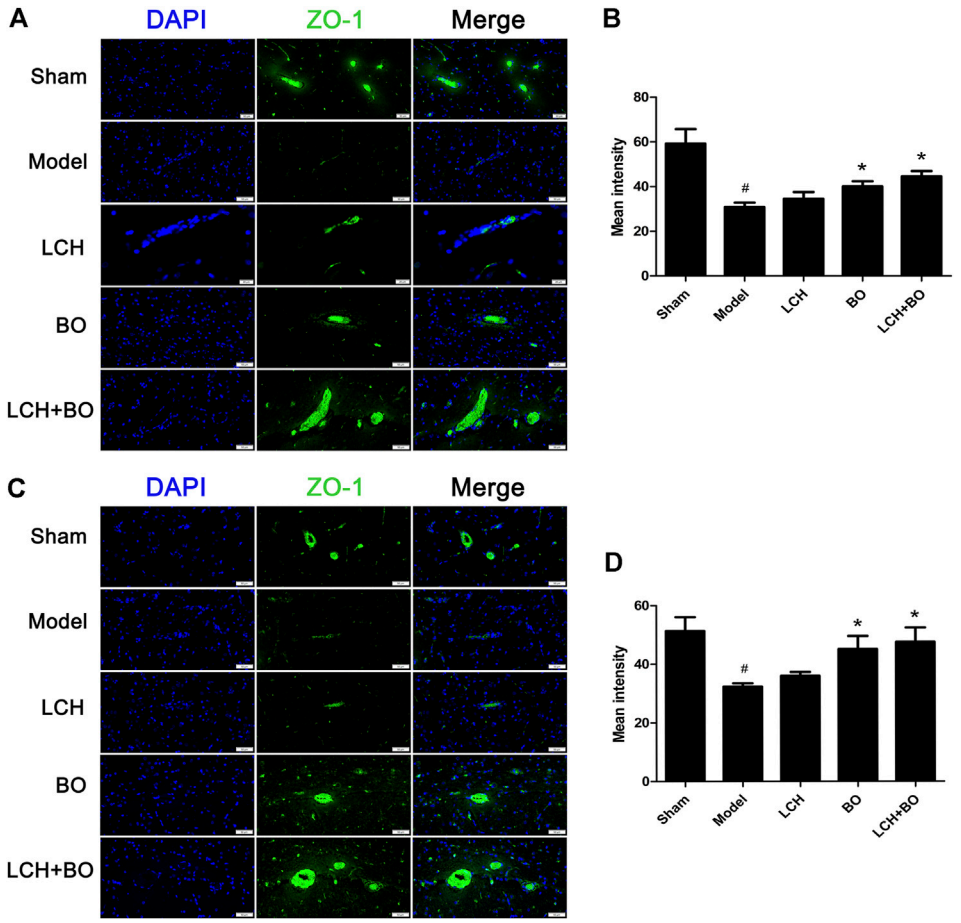

FIGURE 13 | Expressions of ZO-1 within DG and SVZ of MCAO rats. **(A)** Representative immunostaining images of ZO-1 in the DG area. **(B)** The results of average fluorescence intensities of ZO-1 in the DG area (n = 3). **(C)** Representative immunostaining images of ZO-1 in the SVZ area. **(D)** The results of average fluorescence intensities of ZO-1 in the SVZ area (n = 3). $^{\#}p < 0.05$ compared to the sham group; $^{*}p < 0.05$ compared to the model group.

loose, which indicated that BBB structures had been damaged to some extent. No obvious pinocytosis was found yet. The BBB ultrastructures of the LCH group were similar to those of the model group.

Under the treatment of BO, the TJs between endothelial cells were restored, and the gaps between endothelium and basement membrane were significantly reduced, which indicated that BBB structures were recovering from the ischemic injury. Interestingly, some pinocytotic vesicles appeared in the cytoplasm near the vascular lumen. The results implied that BO not only improved BBB structures but also promoted pinocytosis, which might help the drugs with small molecule across the BBB and influx into the brain. The LCH + BO group had performance similar to the BO group.

Borneol Provides a Major Effect on Brain Microvascular Endothelial cell Proliferation and Tight Junction–Associated Proteins Expressions

von Willebrand factor is widely regarded as a biomarker of BMECs, which are sealed by TJ-associated proteins, such as claudin-5, JAM-3, occludin, and ZO-1 (Zhao et al., 2020). Compared to the sham group, the expressions of vWF (**Figure 9**), claudin–5 (**Figure 10**), JAM–3 (**Figure 11**), occludin (**Figure 12**), and ZO–1(**Figure 13**) in the model group were all reduced markedly ($p < 0.05$,0.01) in DG and SVZ areas, which indicated the loss of BMECs and deficiencies of TJs. Unlike the effects on neurogenesis, LCH shows no obvious improvement in BMECs and TJs proteins ($p > 0.05$). However, BO displayed surprising inhibitions on the decreases of vWF, claudin-5, JAM-3, occludin, and ZO-1 in both the areas ($p < 0.05$), which verified its advantages in maintaining the BBB structure, and further cleared its mechanisms in reducing BBB permeability and improving ultrastructure above.

Ligusticum chuanxiong Hort and Borneol Display Different Focuses on the Regulations of Neurotrophins

Neurotrophins play key roles in neurogenesis, and some of which are even involved in angiogenesis and BBB maintenance, such as VEGF and bFGF. In the present research, the results in DG

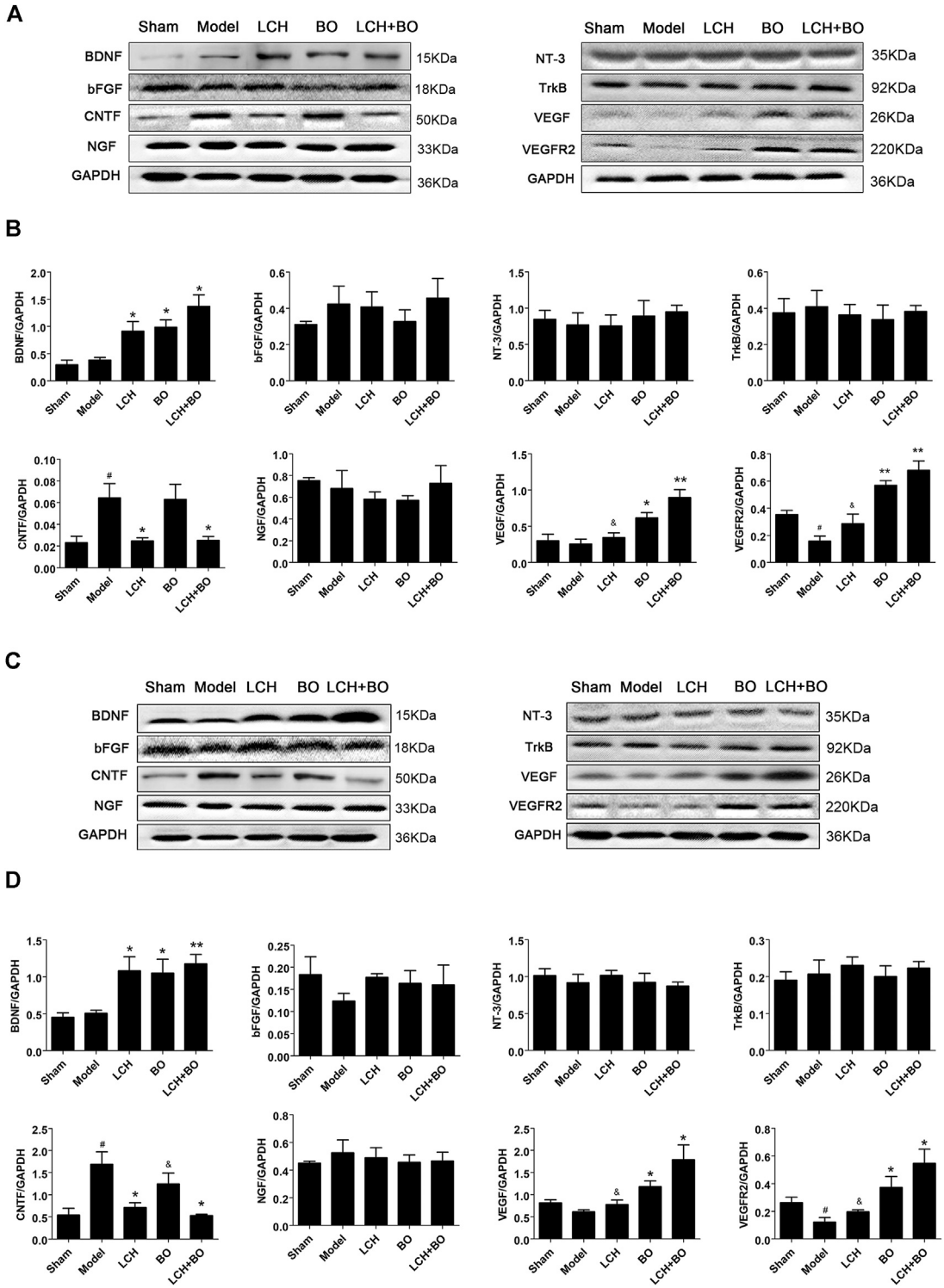

FIGURE 14 | Expressions of BDNF, bFGF, CNTF, NGF, NT-3, TrkB, VEGF, and VEGFR2 within DG and SVZ of MCAO rats. **(A)** Blot images of the proteins in the DG area. **(B)** Relative expression of the proteins to GAPDH in the DG area ($n = 3$). **(C)** Blot images of the proteins in the SVZ area. **(D)** Relative expressions of the proteins to GAPDH in the SVZ area ($n = 3$). $^{\#}p < 0.05$ compared to the sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the model group; $^{\&}p < 0.05$ compared to the LCH + BO group.

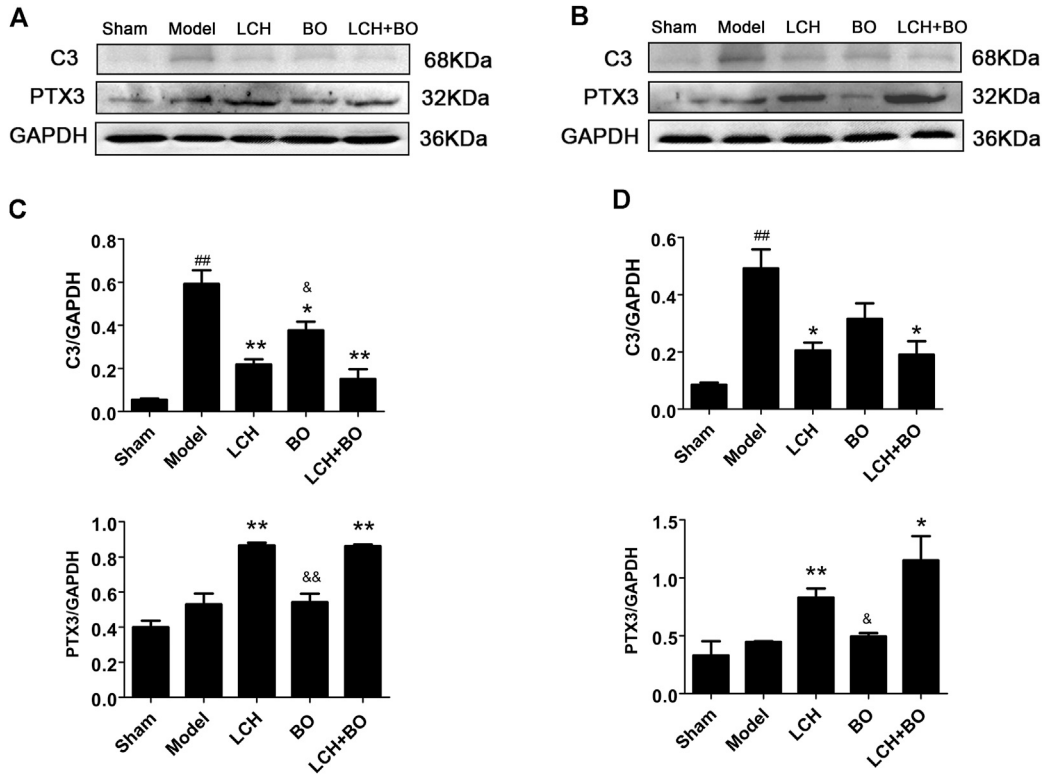

FIGURE 15 | Expressions of C3 and PTX3 within DG and SVZ of MCAO rats. **(A)** Blot images of the proteins in the DG area. **(B)** Blot images of the proteins in the SVZ area. **(C)** Relative expressions of the proteins to GAPDH in the DG area ($n = 3$). **(D)** Relative expressions of the proteins to GAPDH in the SVZ area ($n = 3$). $^{\#\#}p < 0.01$ compared to the sham group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the model group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ compared to the LCH + BO group.

(**Figures 14A,B**) were basically similar to those in SVZ (**Figures 14C,D**), except differences in degree. The increased CNTF and decreased VEGFR2 were shown in DG and SVZ areas of the model group ($p < 0.05$). LCH increased the expression of BDNF and decreased that of CNTF, while BO increased BDNF, VEGF, and VEGFR2 ($p < 0.05$, 0.01). Basically, the combined treatment had a superposition effect of their monotherapies, including increases of BDNF, VEGF, and VEGFR2, and decreases of CNTF. But the improvement of VEGF in the LCH + BO group ($p < 0.01$) was better than that in the BO group ($p < 0.05$), with the condition that LCH showed no effect on the protein ($p > 0.05$), which displayed their synergy to some degree.

Ligusticum chuanxiong Hort is the Main Contributor in Reversing Astrocytes From A1 Phenotype to A2

C3 and PTX3 are widely used as the markers of A1 and A2 ACs, respectively (Su et al., 2019). The present research showed that ischemic injury induced the increases of C3 in both DG (**Figure 15A**) and SVZ (**Figure 15B**) areas ($p < 0.01$). LCH not only decreased C3 but also enhanced PTX3 in the two regions (**Figures 15C,D**) ($p < 0.01$). Surprisingly, BO also decreased C3 level in DG ($p < 0.05$). The therapeutic output of the combined treatment was similar to that of the LCH group. The results indicated that LCH provided the main regulation in reversing reactive ACs from A1 phenotype to A2 during the combined therapy.

DISCUSSION

According to the theory of traditional Chinese medicine, the common therapeutic principle of ischemic stroke is removing blood stasis and inducing resuscitation (Huayu Kaiqiao). LCH and BO are the representative medicines of above therapies, respectively, and widely used for cerebral ischemia patients as a combination, such as Huatuo Zaizao formula and Naoxintong formula (Su et al., 2011) (Duan et al., 2017). Our previous studies also confirmed their synergy in alleviating the loss of neurons and damage of BMECs in cerebral ischemic rats (Yu et al., 2020a; Yu et al., 2020b). However, it is still unclear whether the excellent output of their combined treatment is related to their synergic regulations on neurogenesis and BBB maintenance.

Uncoordinated movement of the limbs or trunk is a typical symptom of stroke, and this abnormal behavior is positively correlated with the degree of neuron injury (Tanaka et al., 2018). In the present study, single LCH treatment markedly enhanced NSS, which exhibited the advantage of LCH in restoring neurological output. Although both LCH and BO significantly reduced infarct ratios and elevated Nissl scores, their

combination displayed a much better effect than their monotherapies on the above indexes. Generally, it is difficult for the combination of drugs with a similar mechanism to bring much better therapeutic effect than their monotherapies. Thus, we inferred that there might be different mechanisms between LCH and BO on their attenuation of ischemic injury. Then the following results verified our hypothesis.

In the results of immunofluorescence measurements, LCH obviously increased the ratio of $Nestin^+/BrdU^+$, which indicated that it upregulated the proliferative potential of NSCs. Moreover, LCH increased $DCX^+/BrdU^+$, DCX^+/DAPI, and $NeuN^+$/DAPI, and decreased $GFAP^+$/DAPI, which indicated that LCH might be involved in inhibiting the excessive reactive astrogliosis, and modulating neurogenesis by guiding the differentiation of NSCs toward neurons, instead of ACs. Additionally, LCH also exerted its neuroprotective effect by alleviating the loss of mature neurons, which is similar to previous reports (Cheng et al., 2008; Gim et al., 2013; Ip et al., 2016; Gu et al., 2020). These above evidences suggested that LCH had the potential of promoting neurogenesis, which might be its new mechanism in ameliorating ischemic brain injury.

BO itself showed little effect on NSC proliferation and neurogenesis in this study. Nevertheless, the regulations on DCX^+/DAPI, $NeuN^+$, $NeuN^+$/DAPI, and $GFAP^+$ were further augmented in the LCH + BO group than those in the LCH group. Although BO has been confirmed to be a p-glycoprotein inhibitor and increases distributions of many drugs in the brain (Yu et al., 2013a; Yu et al., 2013b), its brain-targeting effect is meaningless when the BBB structure is destructed during cerebral ischemia. In view of this, there might be other underlying mechanisms on its neurogenesis assistance for LCH. There have been numerous reports verifying that BO is able to reduce the permeability of the BBB suffering from ischemic injury (Ni et al., 2011; Zhang et al., 2017b). However, the exact mechanism is unknown till now. The present study found that BO decreased the delivery of EB in ischemic brain, as reported previously (Chen et al., 2019). Consequently, the ultrastructure results showed that BO not only reinforced structures of the BBB TJs but also gave rise to pinocytosis, which might be another mechanism of BO in helping drugs across the BBB. The BBB is composed of three cellular elements, including endothelial cells, astrocyte end feet, and pericytes. But only endothelial cells are considered to be the most important element in the BBB structure because TJs are regarded as a series of fusion points between the membrane of adjacent cells and regulated by TJ-associated proteins, such as claudin-5, JAM-3, occludin, and ZO-1 (Ballabh et al., 2004). The protein of vWF is usually used as a biomarker of BMECs. In the model group, the downregulation of vWF indicated the loss of BMECs, which reversely led to the damage of the BBB structure. The expression of vWF was increased, and the ultrastructure of TJs was restored in both DG and SVZ areas after BO treatment. The results indicated that BO had the ability to induce the proliferation of BMECs and even repair the damaged structure of TJs, which might be a potential mechanism of BO in improving the BBB of ischemic brain. For further exploring the statuses of TJs, the immunofluorescence intensities of TJ-associated proteins were detected. Basically, the expressions of those proteins were similar between DG and SVZ areas. Ischemic attack induced the downregulations of claudin-5, JAM-3, occludin, and ZO-1, which implied the decreases of fusion points between the membrane of adjacent BMECs. Then the increased distance between adjacent BMECs caused the collapse of the BBB structure. While the abundances of claudin-5, JAM-3, occludin, and ZO-1 were all increased after BO treatment, they showed no obvious elevation in the LCH group. Apparently, BO played a more crucial role than LCH in the maintenances of the BBB structure and TJ function during the combined therapy.

It has been verified that neurotrophins play a vital role in NSC proliferation, neurogenesis, neuroplasticity, and even BBB remodeling (Maejima et al., 2019; Yang et al., 2020). The present study showed that both LCH and BO upregulated the expression of BDNF. Furthermore, LCH induced the decrease of CNTF, while BO led to the increases of VEGF and VEGFR2. However, the other neurotrophins showed no obvious alterations. BDNF, as a member of neurotrophin family, produces its physiological effect *via* binding to its receptor TrkB, dimerization of ligand and receptor, and autophosphorylation (Yang et al., 2020). There was no marked alteration on the expression of TrkB after the treatment of LCH or BO, which indicated that phosphorylation might be involved in its modulation, instead of abundance. VEGFR2 (KDR/Flk-1), with the structures of seven extracellular immunoglobulin homology domains, a transmembrane domain, and a tyrosine kinase domain, is considered to be the most important receptor of VEGF in regulating physiological and pathological angiogeneses (Takahashi et al., 1999; Simons et al., 2016; Nascimento et al., 2021). Unlike TrkB, the present research proved that the abundances of VEGFR2 and its ligand of VEGF could both be enhanced by BO in DG and SVZ areas, which might be its mechanism involved in BBB remodeling *via* angiogenesis.

Normally, ACs act as important components of neurogenic microenvironment and play an important part in neuronal maturation and function. However, stroke attack causes the loss of neurons, excessive proliferation of ACs, and even formation of glial scar within ischemic area. Reactive astrogliosis is regarded as the main source of glial scar. CNTF is able to lead to sustained AC activation, which produces multifaceted functions in CNS pathogenesis process, including beneficial and harmful aspects. However, the benefits of reactive ACs at the acute phase of stroke might be offset by its potential of negatively regulating neurogenesis at a later phase (Wilhelmsson et al., 2012). A previous study confirmed that MCAO injury led to transient increase of ACs with A2 phenotype, followed by rapid reduction, while inflammation caused increase of A1 ACs (Zamanian et al., 2012). Additionally, inflammatory reaction accompanied and aggravates the development of ischemic pathology, especially during the later stage of stroke (Kawabori and Yenari, 2015). In the present study, model rat showed significant increases of GFAP and C3, which indicated the activation of excessive A1 ACs by CNTF at the later stage of stroke, while LCH not only alleviated the activation to a certain extent *via* reducing the expression of CNTF but also guided the transformation of reactive ACs from A1 to A2, which reversely promoted the release of some neurotrophic factors, such as BDNF. Furthermore, BO also reduced reactive astroliosis with A1

phenotype *via* maintaining homeostatic intracerebral environment. Apparently, both LCH and BO had the ability of regulating ACs phenotypes, but the involved mechanisms were different.

Presently, the treatment of ischemic stroke is still limited in clinic. Inducing neurogenesis and restoring the damaged neurological function, as potential strategies, share more and more concerns. The results in this study indicated that the synergy between LCH and BO is mainly based on neurogenesis *via* transformation of AC phenotypes, modulation of neurotrophins, proliferation of NSCs, and maintenance of the BBB. Although the present study verified the underlying mechanism of LCH on neurogenesis, the main active ingredients and the synergic mechanism among these ingredients are still unclear, and seeking answers to these questions is our future work.

CONCLUSION

In the present study, the synergic therapies between LCH and BO were shown on MCAO rats, including NSS, infarct areas, and Nissl scores. However, these two medicines displayed different focuses. Specifically, LCH addressed the regulations on NSC proliferation, neurogenesis, mature neurons protection, and AC transformation from A1 phenotype to A2, which then regulated the expressions of CNTF and BDNF. But BO was mainly responsible for maintaining the integrity of the BBB, including remolding structures of TJs and upregulating TJ-associated proteins. Additionally, the results also indicated that a homeostatic intracerebral environment played a crucial role in post-stroke neuroregeneration.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine.

AUTHOR CONTRIBUTIONS

BY and LX designed research route; BY, YY, XZ, MR, and JL performed the experiments; BY, YY, XZ, and ZZ analyzed data; BY, YY, and TL prepared the draft; BY approved the final version. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.666790/full#supplementary-material

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REVIEW
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Protective Effects and Network Analysis of Ginsenoside Rb1 Against Cerebral Ischemia Injury: A Pharmacological Review

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Ischemic stroke is a leading cause of death and disability worldwide. Currently, only a limited number of drugs are available for treating ischemic stroke. Hence, studies aiming to explore and develop other potential strategies and agents for preventing and treating ischemic stroke are urgently needed. Ginseng Rb1 (GRb1), a saponin from natural active ingredients derived from traditional Chinese medicine (TCM), exerts neuroprotective effects on the central nervous system (CNS). We conducted this review to explore and summarize the protective effects and mechanisms of GRb1 on cerebral ischemic injury, providing a valuable reference and insights for developing new agents to treat ischemic stroke. Our summarized results indicate that GRb1 exerts significant neuroprotective effects on cerebral ischemic injury both *in vivo* and *in vitro*, and these network actions and underlying mechanisms are mediated by antioxidant, anti-inflammatory, and antiapoptotic activities and involve the inhibition of excitotoxicity and $Ca2^{+}$ influx, preservation of blood–brain barrier (BBB) integrity, and maintenance of energy metabolism. These findings indicate the potential of GRb1 as a candidate drug for treating ischemic stroke. Further studies, in particular clinical trials, will be important to confirm its therapeutic value in a clinical setting.

Keywords: ginsenoside Rb1, ischemia and reperfusion injury, ischemia stroke, anti-inflammatory, antioxidant, antiapoptosis

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INTRODUCTION

When the brain becomes blocked by a blood clot and blood is prevented from reaching the brain, an ischemic stroke occurs. Ischemic stroke, approximately accounting for 85% of all diagnosed strokes, has the characteristics of high morbidity, high mortality, high disability, and high recurrence rates and is mainly caused by cerebral ischemia and reperfusion (I/R) injury (CIRI) (Turley et al., 2005; Eltzschig and Eckle, 2011). I/R is a pathological condition characterized by an initial restriction of blood supply to an organ followed by the subsequent restoration of perfusion and concomitant reoxygenation (Turley et al., 2005; Eltzschig and Eckle, 2011), which is one of the leading causes of death worldwide (Turley et al., 2005; Woodruff et al., 2011; Feigin et al., 2014). I/R injury mainly includes ischemic stroke, acute kidney injury, and myocardial infarction. Due to the interrupted

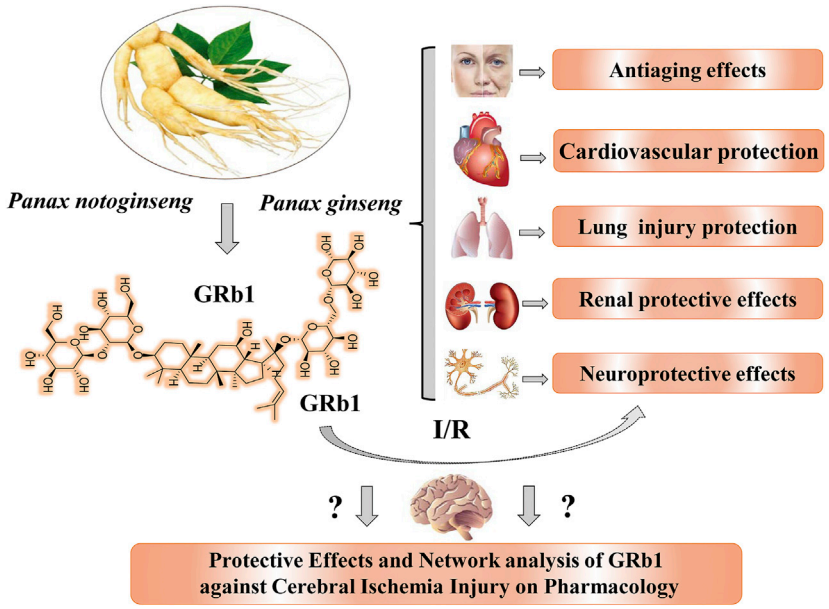

FIGURE 1 | Natural sources, chemical structure, and main pharmacological activities of ginsenoside Rb1 obtained from *P. notoginseng* and *P. ginseng*. GRb1 exerts significant neuroprotective effects, but its efficiency and action network must be further explored and analyzed. GRb1, ginsenoside Rb1; I/R, indicates ischemia and reperfusion.

blood supply to the CNS, cerebral infarction causes ischemic stroke and other forms of CNS injury. As these mechanisms often involve complex combinations of necrosis, apoptosis, necroptosis, and autophagy, ischemic stroke–related pathogenesis is not totally clear (Turley et al., 2005; Eltzschig and Eckle, 2011). Based on accumulating evidence, once an ischemic stroke occurs, the insufficient blood supply directly stimulates neurons, causes the accumulation of glutamate, overactivates a plethora of downstream signaling pathways, and increases the intracellular calcium concentration, which triggers energy metabolism disorders (Bolaños et al., 2009), oxidative stress (Chamorro et al., 2016), Ca^{2+} overload, excitatory neurotransmitter release, the immune-mediated inflammatory response following acute ischemic stroke, and its related apoptosis and necrosis processes (Kamel and Iadecola, 2012; Chamorro et al., 2016), finally resulting in ischemic infarction of the brain. Hence, the main aim of acute stroke treatment is to salvage the ischemic penumbra or volume of hypoperfused, nonfunctional, yet still viable tissue surrounding the infarcted core.

Currently, tissue plasminogen activator (TPA) is regarded as the main effective pharmacological therapy and drug for ischemic stroke (Turley et al., 2005; Woodruff et al., 2011; Chamorro et al., 2016). Scholars have conducted extensive studies and developed some neuroprotective drugs and other interventions for treating ischemic injury (Tuttolomondo et al., 2011; Woodruff et al., 2011), ranging from pharmacologically blocking neurotransmitter receptors to intercepting cell death pathways, as well as the induction of hypothermia or hyperoxygenation. However, most of these studies failed to show the efficacy of any of these promising strategies. The current existing neuroprotective drugs remain limited and insufficient for the clinical treatment needs for ischemic stroke (Turley et al., 2005; Woodruff et al., 2011; Chamorro et al., 2016). Therefore, the development of novel therapeutic strategies and agents for preventing and treating ischemic stroke is urgently needed.

Panax ginseng C. A. Mey and *P. notoginseng* (Burk) F. H. Chen are commonly used as natural medicinal plants in TCM (Hui et al., 2010; Bao-Ying et al., 2014; Yang et al., 2014; Xie et al., 2018a; Xie et al., 2018b), the roots and medicinal ingredients of which have been in use for several hundred years. Pharmacological studies have shown that *P. notoginseng*, *P. ginseng*, and their extracts have many functions (Mancuso and Santangelo, 2017; Kim, 2018), such as anti-inflammatory activity (Allison and Ditor, 2014; Zheng et al., 2014; Zhang et al., 2015; Jeon and Kim, 2016), antioxidant activity, blood glucose regulation (Xie et al., 2016; Zhang et al., 2016), insulin resistance improvement (Zhang and Jiang, 2012; Zhai et al., 2018), inhibition of neuronal apoptosis (Li et al., 2014; Hou et al., 2017; Yang et al., 2017), and neuroprotection (Xie et al., 2018a; Xie et al., 2018b; Wang et al., 2016; Berge and Riise, 2015). Hence, one of the main tasks is to identify natural active substances and compounds that can be utilized for the prevention and treatment of ischemic stroke.

Ginseng Rb1 (GRb1) is a ginsenoside glycol (Liu et al., 2015; Ju et al., 2019) and one of the main active ingredients of *P. notoginseng* and *P. ginseng* (**Figure 1**). GRb1 has been proven to exert significant protective effects on the CNS, cardiovascular system (Xie et al., 2018a; Xie et al., 2018b), and immune system and possesses antitumor activities (Zhou et al., 2018; Zhou et al., 2019a; Zhou et al., 2019b). As shown in **Figure 1**, GRb1 possesses various pharmacological activities, including neuroprotective

(Yang et al., 2008; Liu et al., 2013; Huang et al., 2015; Huang et al., 2015; Ye et al., 2019), acute renal injury-protective (Wang et al., 2008; Sun et al., 2012; Chen et al., 2019), cardiovascular-protective (Wang et al., 2008; Xia et al., 2011; Zheng et al., 2017; Li et al., 2020), lung injury-protective (Wang et al., 2013; Chen et al., 2014; Jiang et al., 2015; Li et al., 2015), and antiaging (Dong et al., 2017; Zhou et al., 2019a) effects, in many *in vitro* and *in vivo* models.

Currently, accumulated experiments and data suggest that GRb1 exerts neuroprotective effects both *in vivo* and *in vitro* and has a great potential as a novel candidate agent for ischemic stroke. To date, researchers have not clearly determined whether GRb1 can be used to treat ischemic stroke and cerebral I/R injury. No systematic review or analysis has been conducted to assess the protective effects and mechanisms by which GRb1 combats ischemic stroke and I/R injury (**Figure 1**). Hence, we conducted this analysis of preclinical studies of the effect of GRb1 on ischemic stroke. We searched the PubMed and China National Knowledge Infrastructure databases *via* using "Ginsenoside Rb1" and "Ischemia" as search terms. The PubMed database was comprehensively searched up to September 2020, and it showed 66 literatures; we excluded some irrelevant ones and then divided them into several aspects to further analyze the pharmacological effects and mechanisms of GRb1 in pre-treating and treating CIRI. Furthermore, we manually searched for other potential and relevant references, and there were no limitations in the language of all publications.

In this review, we found that GRb1 might alleviate cerebral/neural ischemia injury *via* its antiapoptotic, antioxidant, and anti-inflammatory activities, and effects on mitochondrial homeostasis, promoting neurogenesis, and improving brain functional connections and interactions, which provides more evidence for basic studies and further promotes the development of GRb1 as a candidate drug for the clinical treatment of ischemic stroke.

ANTIAPOPTOTIC EFFECTS AND NEURONS

Recent reports have suggested that GRb1, a natural saponin ingredient of TCM, exerts remarkable neuroprotective effects on the CNS (Ahmed et al., 2016), prevents and alleviates cerebral I/R injury *via* anti-inflammatory activity, antioxidant activity, enhanced neuroproliferation and neurodifferentiation, and improved energy metabolism (Chen et al., 2010; Zhu et al., 2012; Huang et al., 2015), confirming that Rb1 exerts antiapoptotic effects on neurons.

First, *in vivo* experiments showed that Rb1 significantly inhibited CA1 neuronal death caused by a 2-vessel occlusion (2-VO) model in rats (Luo et al., 2014) and delayed neuronal death in gerbils (Lim et al., 1997); noticeably reduced the infarct size and neuronal deficits, relieved pathological changes (Yuan et al., 2007), and decreased the number of neural apoptotic cells (Wen et al., 1996) in rats with middle cerebral artery occlusion (MCAO) (Lim et al., 1997; Yuan et al., 2007; Yang et al., 2008); and scavenged free radicals (Lim et al., 1997) and improved hippocampal blood flow at 5 min after transient forebrain ischemia (Lim et al., 1997). Rb1 significantly prolonged the response latency of ischemic gerbils and rescued a significant number of ischemic CA1 pyramidal neurons (Wen et al., 1996; Lim et al., 1997). Under abnormal ischemic microenvironmental conditions, Rb1 evidently decreased the concentrations of glutamic acid and Ca^{2+} (Wang et al., 2017), noticeably alleviated memory deficits in rats, and reduced pyramidal cellular necrosis and apoptosis in the hippocampus induced by glutamate (Glu) and Ca^{2+} (Wang et al., 2017; Guo et al., 2018). Additionally, Rb1 suppressed the loss of BBB integrity by suppressing the induction of neuroinflammation in a model of ischemic stroke (Chen et al., 2015). Based on these results, Rb1, an effective neuroprotective drug, plays key roles in cerebral I/R injury.

Second, *in vitro* experiments (**Table 1**) suggested that Rb1 might increase cell viability, inhibit oxygen and glucose deprivation (OGD)–induced neuronal death, and reduce autophagic vacuoles in SH-SY5Y cells (Luo et al., 2014), changes that were blocked by the inhibitor LY294002. GRb1 significantly decreased the levels of free radicals, protected hippocampal neurons from lethal damage caused by the hydroxyl radical–promoting agent $FeSO_4$ *in vitro* (Lim et al., 1997), and markedly suppressed the uptake of Glu and overload of Ca^{2+} in OGD-induced SH-SY5Y cells (Wang et al., 2017; Guo et al., 2018). GRb1 also significantly reduced the levels of lactate dehydrogenase (LDH) (Park et al., 2005; Li et al., 2016), nitric oxide (NO), and superoxide (O-) (Zhu et al., 2012; Huang et al., 2014). Thus, Rb1 might be regarded as an antiapoptotic agent with neuroprotective effects.

Furthermore, GRb1 significantly inhibited the expression of the proapoptotic genes Bax, Bad, and caspase-3 (Yang et al., 2008; Gao et al., 2010), upregulated Bcl-2 and the ratio of Bcl-2/Bax *in vivo* and *in vitro* (Yuan et al., 2007; Yang et al., 2008), increased the expression of glial-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) (Yuan et al., 2007; Gao et al., 2010), and thus prevented neuronal death induced by cerebral ischemia. GRb1 significantly increased the levels of Akt phosphorylated at Ser473 (P-Akt) and reduced the expression levels of LC3II and Beclin1, indicating that GRb1 might prevent ischemic neuronal death by modulating autophagy activation (Luo et al., 2014); moreover, GRb1 increased the levels of P-Akt and P-mTOR, reduced P-PTEN levels *in vivo* and *in vitro*, and ameliorated the abnormal microenvironment by activating the P-AKT/P-mTOR pathway and inhibiting P-PTEN (Guo et al., 2018). In contrast, the LY294002 treatment reversed these changes induced by GRb1. Furthermore, GRb1 inhibited the expression of NMDAR, increased the expression of glial glutamate transporter 1 (GLT-1), and downregulated the levels of cytochrome C (Cyt-C) in response to neuronal mitochondrial stress, which reduced the excessive Glu and Ca^{2+} levels (Wang et al., 2017; Guo et al., 2018).

In general, evidence from recent studies suggests that GRb1 may exert its antiapoptotic effects by regulating the phosphoinositide 3-kinase (PI3K)-protein kinase B (AKT)-TOR signaling pathways, inhibiting autophagy, alleviating mitochondrial stress and apoptosis pathways, modulating the expression of N-methyl-D-aspartate-receptor (NMDAR) and

TABLE 1 | Antiapoptotic effects of GRb1 on cerebral I/R injury based on current reports and results.

Type	Animal and dose	Model	Effect	Mechanism	CF
TGI	SD rat: 20–40 mg/kg SH-SY5Y: 1–10 μM	2-VO model OGD/R	↑ Cellular viability ↓ Neuronal death ↓ Autophagic vacuoles	↓ LC3II and Beclin1 ↑ PI3K/phosphor-Akt LY294002 Verify	Luo et al. (2014)
TCI	SD rats: 40 mg/kg	MCAO	↓ Pathological changes ↓ Apoptotic neural cells	↑ Bcl-2 ↓ Bax	Yang et al. (2008)
TFI	Gerbils: 0.09–90 fM Hippocampal neurons	MCAO FeSO4 treatment	↓ Free radicals ↑ response latency ↑ Hippocampal CA1 neurons	↓ Oxidative damage ↓ Apoptosis	Lim et al. (1997)
TCI	SD rats: 40 mg/kg	MCAO	↓ Infarct and neuronal deficit ↓ Apoptotic cells	↑ GDNF ↑ Bcl-2	Yuan et al. (2007)
TCI	Gerbils: 10–20 mg/kg	MCAO	↑ response latency and synapses ↓ Pyramidal neurons	↓ Apoptosis	Wen et al. (1996)
TCI	SD rats: 40 mg/kg	MCAO	↑ Neurological functions ↑ Nestin-positive cells	↑ BDNF ↓ Caspase-3	Gao et al. (2010)
TGI	ICR mice: 5–40 mg/kg	MCAO	↓ Infarction and brain edema ↓ EB extravasation ↑ BBB integrity ↓ MMP-9, IL-1β, and NO synthase	↑ Arginase 1 and IL-10 ↓ NOX-4 and NOX ↓ Free radicals Neuroinflammation	Chen et al. (2015)
IAM	SD rats: 25–100 mg/kg SH-SY5Y cells: 10 μM	OGD/R Microperfusion	↓ Memory deficit pyramidal ↓ Necrosis and apoptosis ↓ Glu and Ca^{2+}	↑ P-Akt/P-mTOR ↓ P-PTEN Akt/mTOR/PTEN	Guo et al. (2018)
IAM	SD rats: 40 mg/kg	Microperfusion	↑ rCBF and GLT-1 ↑ Neuronal ultrastructure ↓ Glu and overload of Ca^{2+}	↓ NMDAR and Cyt-C ↓ Neuronal mitochondrial damage	Wang et al. (2017)

TGI, transient global ischemia; 2-VO, 2-vessel occlusion model; TCI, transient cerebral ischemia; TFI, transient forebrain ischemia; IAM, ischemic abnormal microenvironment; MCAO, middle cerebral artery occlusion; OGD/R, oxygen–glucose deprivation/reperfusion; GDNF, glial-derived neurotrophic factor; NO, nitric oxide; Glu, glutamate; MMP-9, matrix metalloprotein 9; IL, interleukin; BBB, blood–brain barrier; BDNF, brain-derived neurotrophic factor; rCBF, regional cerebral blood flow; GLT-1, glial glutamate transporter1; Cyt-C, cytochrome C; SD, Sprague–Dawley; NOX, NADPH oxidase; EB, Evans blue; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; NMDAR, N-methyl-D-aspartate-receptor; CF, cited references.

GLT-1, and improving neurogenesis and BDNF levels. Nonetheless, currently, the mechanisms by which GRb1 regulates ischemic neuronal apoptosis are not completely elaborated and summarized and should be further explored in the future.

REGULATION OF NEUROINFLAMMATION AND MICROGLIA

Although CIRI is a complex pathology caused by the interaction of numerous pathophysiological factors (Allison and Ditor, 2014; Anderson et al., 2014), accumulating evidence indicates that acute inflammation and subsequent apoptosis and necrosis are involved in the progression of a cerebral ischemic insult (Jianhua et al., 2008; Eltzschig and Eckle, 2011; Jiang et al., 2014; Tao et al., 2015). Recently, GRb1 was reported to exert beneficial effects on cerebral ischemic stroke and to inhibit inflammatory cascades in the acute phases of cerebral ischemia (Wang et al., 2008; Zhu et al., 2012; Jiang et al., 2015).

According to recent studies (**Table 2**), GRb1 noticeably reduces the infarct volume, significantly alleviates neurological deficits, decreases neurological severity scores (Zhu et al., 2012; Liu et al., 2013; Liu et al., 2018; Chen et al., 2020), preserves the neuronal morphology and structure (Ke et al., 2014), improves pathological changes (Liu et al., 2018), and inhibits the activation of microglia in MCAO/R model rats (Zhu et al., 2012) and N9 microglia *in vitro* (Ke et al., 2014). These neuroprotective effects may be involved in microglia-mediated CNS inflammation and related neuronal damage in the acute phases of cerebral ischemia/hypoxia.

On the one hand, ginsenoside GRb1 notably decreased the activation of microglia induced by I/R (Zhu et al., 2012) and a hypoxic coculture system (Ke et al., 2014) and significantly reduced the levels of the interleukin (IL)-1β (Zhu et al., 2012; Liu et al., 2013; Chen et al., 2015; Chen et al., 2020), tumor necrosis factor-α (TNF-α) (Zhu et al., 2012; Ke et al., 2014; Liu et al., 2018; Chen et al., 2020), IL-6 (Zhu et al., 2012; Liu et al., 2018; Chen et al., 2020), and high mobility group protein 1 (HMGB1) mRNAs and proteins in brain tissue and serum (Liu et al., 2018); on the other hand, GRb1 significantly decreased the levels of NO (Chen et al., 2015), superoxide (Ke et al., 2014; Liu et al., 2018), and nitric oxide synthase (Liu et al., 2018) produced by microglia *in vitro* (Ke et al., 2014) and *in vivo* (Zhu et al., 2012; Liu et al., 2018), reduced neuronal apoptosis (Ke et al., 2014; Liu et al., 2018), and reduced the levels of cleaved caspase-3 and caspase-9 (Ke et al., 2014; Liu et al., 2018).

Further studies revealed that GRb1 inhibited nuclear factor kappa B (NF-κB) signaling pathways by decreasing the levels of phosphorylated NF-κB/p65 and IB-kinase complex (IKK) and downregulating HMGB1 and its related local inflammation (Ke et al., 2014; Liu et al., 2018), which stimulate NF-κB translocation and mitogen-activated protein kinase (MAPK) phosphorylation triggered by HMGB-1/TLR4 (Lu et al., 2011; Procaccio et al., 2014; Sun and Nan, 2016; Xie et al., 2019). Moreover, GRb1 reduced the expression levels of proinflammatory factors,

TABLE 2 | Neuroprotective effects of GRb1 on cerebral ischemia injury are mediated by suppressing neuroinflammation and microglia-mediated inflammatory reactions, based on current reports and results.

Type	Animal and dose	Model	Effect	Mechanism	CF
In vivo TCI	SD rats: 40 mg/kg	MCAO	↓ TNF-α, IL-6 ↓ Activation of microglia	↓ p-NF-κB/p65 ↓ NF-κB pathway	Zhu et al. (2012)
In vitro	Cortical neurons N9 microglia	Hypoxic co-culture	↑ Cell viability ↑ Neuronal morphology ↓ NO, superoxide, and TNF-α	↓ Neuronal apoptosis ↓ Caspase-3 and microglia ↓ Inflammatory reaction	Ke et al. (2014)
In vivo TCI	SD rats: 40 mg/kg	MCAO	↓ Neurologic defect degree ↓ Cerebral infarction volume	↓ IL-1β ↓ Inflammatory damage	Liu et al. (2013)
In vivo TCI	SD rats: 50–100 mg/kg	MCAO	↓ TNF-α and IL-6 ↓ Infarct volume ↓ Neuronal apoptosis ↓ NO synthase and NO	↓ NF-κB pathway ↓ Cleaved caspase-3 ↓ Caspase-9 and HMGB1	Liu et al. (2018)
In vivo TGI	ICR mice: 5–40 mg/kg	MCAO	↑ BBB integrity ↓ EB extravasation ↓ Infarction and brain edema ↓ MMP-9, IL-1β, and NO synthase	↓ Free radicals ↑ Arginase 1 and IL-10 ↓ NOX-4 and NOX ↓ Neuroinflammation	Chen et al. (2015)
In vivo TGI	SD rats: 50 mg/kg Probiotic	Pseudo germ-free, MCAO	↓ Infarct size ↓ Neurological deficit score ↓ IL-1β, IL-6, and TNF-α	↑ GABA ↑ Probiotics *Lac.H* ↑ GABA receptors	Chen et al. (2020)

TGI, transient global ischemia; TCI, transient cerebral ischemia; IL, interleukin; SD, Sprague–Dawley; MCAO, middle cerebral artery occlusion; TNF-α, tumor necrosis factor α; NF-κB, nuclear factor-κB; NO, nitric oxide; MMP-9, matrix metalloprotein 9; BBB, blood–brain barrier; NOX, NADPH oxidase; EB, Evans blue; HMGB1, high mobility group protein-1; CF, cited references.

attenuated the activity of MMP-9 (Chen et al., 2015), upregulated the expression of γ-aminobutyric acid (GABA) receptors in I/R rats (Chen et al., 2020), protected BBB integrity in ischemic stroke by suppressing neuroinflammation, decreased the production of MMP-9 and NOX4-derived free radicals (Chen et al., 2015), and regulated the probiotic *Lac.H* and GABA receptor levels (Chen et al., 2020). Overall, GRb1 may exert a neuroprotective effect on I/R-induced injury by suppressing neuroinflammation and microglia-mediated inflammatory reactions.

ANTIOXIDANT EFFECTS AND MITOCHONDRIA

Oxidative stress plays an important role, and reactive oxygen species (ROS) is implicated in the tissue damage that occurs in cerebral ischemic pathogenesis, which results in the production of toxic molecules that alter cellular proteins, lipids, and ribonucleic acids, leading to cell dysfunction or death (Crack and Taylor, 2005; Chamorro et al., 2016). Excessive ROS severely impair mitochondria and their related energetic metabolism functions. GRb1 exhibits various pharmacological activities, including antioxidant (Dong et al., 2017; Li et al., 2019; Xu et al., 2019; Ye et al., 2019), antiapoptotic, and neuroprotective properties.

First, the results of *in vivo* experiments (**Table 3**) showed that GRb1 protected hippocampal CA1 neurons (Lim et al., 1997), reduced free radicals (Chen et al., 2015), and possessed potential for treating brain injuries (Yang et al., 2008); meanwhile, GRb1 increased superoxide dismutase (SOD) activity, decreased malondialdehyde (MDA) contents in the serum and spinal cord tissue (Ye et al., 2019), and inhibited oxidative stress and extracellular signal-regulated kinase (ERK) activation in aged mice exposed to cerebral ischemia (Dong et al., 2017). All of the antioxidant effects of GRb1 are beneficial for reducing neuronal death, mitochondrial damage, and astrocyte injury induced by I/R, indicating that GRb1 may reduce pathological changes and decrease neural cell apoptosis (Yang et al., 2008; Chen et al., 2015).

Second, *in vitro* experiments (**Table 3**) confirmed that GRb1 significantly improved cell viability, decreased intracellular ROS production, and increased catalase (CAT) activity and the mtDNA copy number in OGD/R-induced astrocytes, thus inhibiting oxidative phosphorylation (OXPHOS) (Xu et al., 2019). GRb1 exerted obvious protective effects on the mitochondria by distinctly attenuating MMP depolarization, improving the efficiency of mitochondrial OXPHOS, increasing the activities of complexes I, II, III, and V, and increasing the level of adenosine triphosphate (ATP) following OGD/R induction (Xu et al., 2019).

Further studies (**Table 3**) revealed the antioxidant effects of GRb1. On the one hand, GRb1 significantly downregulated NOX-4 expression and NOX activities in ischemic rat brain tissues (Chen et al., 2015; Dong et al., 2017), inhibited ischemia-stimulated NADPH oxidase gene expression, including NOX-1, 2, and 4 (Dong et al., 2017), and prevented ERK activation in aged mice (Dong et al., 2017). On the other hand, GRb1 significantly suppressed mitochondrial damage, increased the mitochondrial membrane potential (MMP) in OGD/R-induced astrocytes (Xu et al., 2019), and thus improved the antioxidant activity (CAT and SOD). GRb1 remarkably increased aquaporin (AQP) 4 levels (Li et al., 2019), decreased Bax expression (Yang et al., 2008), and upregulated Bcl-2 (Yang et al., 2008), nerve growth factor (NGF), and BDNT expression (Li et al., 2019) *in vivo* and *in vitro*. Thus, GRb1 is potentially useful for treating brain injury, due to its antioxidant effects and mitochondrial protection.

TABLE 3 | Antioxidant effects of GRb1 on I/R neuronal injury mediated by the inhibition of oxidative stress and mitochondrial injury, increases in energy metabolism and protection of the BBB, based on recent reports and results.

Type	Animal and dose	Model	Effect	Mechanism	CF
In vitro I/R	SD rats Astrocyte: 6.71–32.00 mg/ml	OGD/R	↓ Spinal cord edema ↑ Neurological function ↓ Cellular membrane permeability	↑ AQP ↑ NGF ↑ BDNF	Li et al. (2019)
In vivo TCI	SD rats: 40 mg/kg	MCAO	↑ Nestin-positive cells ↑ Neurological functions Histological feature	↑ BDNF ↓ Caspase-3 ↑ Promotion of neurogenesis	Gao et al. (2010)
In vitro I/R	C57BL/6J Mice Astrocytes: 10 μM	OGD/R	↓ Intracellular ROS ↑ Cell viability, CAT, and ATP ↑ mtDNA copy number and MMP ↑ Complexes I, II, III, and V	↑ Efficiency of mitochondrial oxidative phosphorylation	Xu et al. (2019)
In vivo TCI	C57 Mice: 0.5–10 mg/kg	MCAO	↓ Brain trauma ↓ NOX-1, -2, and -4 ↓ NADPH oxidase gen	↓ ERK activation ↓ Oxidative stress	Dong et al. (2017)
In vivo TGI	ICR mice: 5–40 mg/kg	MCAO	↑ BBB integrity ↓ EB extravasation ↓ Infarction and brain edema ↓ MMP-9, IL-1β, and NO synthase	↓ Free radicals ↑ Arginase 1 and IL-10 ↓ NOX-4 and NOX ↓ Neuroinflammation	Chen et al. (2015)
In vivo SCII	SD rats: 20–80 mg/kg	SCII	↑ SOD; ↓ MDA ↑ Neurological function	↓ Apoptosis ↑ Survivin protein	Ye et al. (2019)
In vivo SCII	SD rats: 15 mg/kg	SCII	↓ Spinal cord apoptosis ↑ Hindlimb locomotor function	↑ Bcl-2/Bax ratio ↑ Caspase-3 and p-Ask-1	Zhao et al. (2018)
In vivo TCHI	Mongolian gerbils: 250 μg/ml	Occluding BVA	↓ Hearing loss ↓ Auditory brainstem response	↓ Neural cell apoptosis	Fujita et al. (2007)

SCII, spinal cord I/R injury; TGI, transient global ischemia; TCI, transient cerebral ischemia; TCHI, transient cochlear ischemia; SD, Sprague–Dawley; NO, nitric oxide; MMP-9, matrix metalloprotein 9; BBB, blood–brain barrier; NOX, NADPH oxidase; EB, Evans blue; OGD/R, oxygen–glucose deprivation/reperfusion; MCAO, middle cerebral artery occlusion; AQP, aquaporin; NGF, nerve growth factor; BNDF, brain-derived neurotrophic factor; ROS, reactive oxygen species; CAT, catalase; ATP, adenosine triphosphate; MMP, mitochondrial membrane potential; mtDNA, mitochondrial DNA; ERK, extracellular signal-regulated kinase; SOD, superoxide dismutase; MDA, malondialdehyde; BVA, bilateral vertebral arteries; CF, cited references.

INHIBITION OF OTHER TYPES OF NEURAL ISCHEMIC INJURY

Related studies have shown that GRb1 significantly alleviates I/R injury of the kidney (Wang et al., 2008; Sun et al., 2012; Sun et al., 2013; Chen et al., 2019) and brain (Chen et al., 2010; Zhu et al., 2012; Huang et al., 2015). However, few reports have assessed spinal cord ischemia–reperfusion injury (SCII). According to recent studies, GRb1 reduces cell apoptosis induced by SCII by inhibiting oxidative stress (Ye et al., 2019). GRb1 noticeably improves hindlimb locomotor dysfunction in rats (Zhao et al., 2018), increases SOD activity, decreases the MDA content in serum and spinal cord tissue (Ye et al., 2019), and inhibits neuronal apoptosis. The potential mechanisms may be tightly associated with promoting the expression of survivin (Ye et al., 2019), downregulating the levels of caspase-3 and phosphorylated Ask-1 (p-Ask-1), and improving the Bcl-2/Bax ratio (Zhao et al., 2018) in SCII rats. After the exposure of spiral ganglion cells (SGCs)to transient cochlear ischemia, GRb1 significantly reduced the percentage of the auditory brainstem response threshold shift and prevented hearing loss caused by ischemic injury to SGCs in Mongolian gerbils (Fujita et al., 2007), indicating that GRb1 might protect SGCs from ischemic injury.

In addition, GRb1 clearly increased the expression of NGF in OGD/R-stimulated astrocytes (Li et al., 2019), upregulated BDNF expression, and downregulated caspase-3 expression in MCAO/R model rats (Yuan et al., 2007; Gao et al., 2010). Therefore, GRb1 may exert neuroprotective effects by promoting neurogenesis.

ANALYSIS OF SAFETY AND THERAPEUTIC EFFECTIVENESS

Based on the above summarized findings (**Tables 1**–**3**), Rb1 has a potential therapeutic value for ischemic stroke *via* immunological, antioxidant, and neuroprotective effects. However, the efficacy and safety of Rb1 for ischemic stroke in human have not been tested, and its effective dose is not known. Nevertheless, there is evidence that red ginseng extract, which contains 20–30% of GRb1, at 23, 25, 50, and 100 mg with repeated oral administration for 1–2 weeks, is safe and well tolerant in healthy subjects (Kim, 2013; Jin et al., 2019; Choi et al., 2020). In addition, randomized controlled studies showed that 200–400 mg of ginsenosides or its preparations significantly ameliorated neurological deficit (He et al., 2011) and improved working memory and daily activities of patients with ischemic stroke (Reay et al., 2010; He et al., 2011), indicating that a daily dose of 40–120 mg of GRb1 may be safe and effective for treating ischemic stroke. The effective dose of Rb1 tested in animal studies with notably attenuated ischemia-induced cerebral I/R injury ranges from 10 to 40 mg/kg (**Tables 1**–**3**), which is equivalent

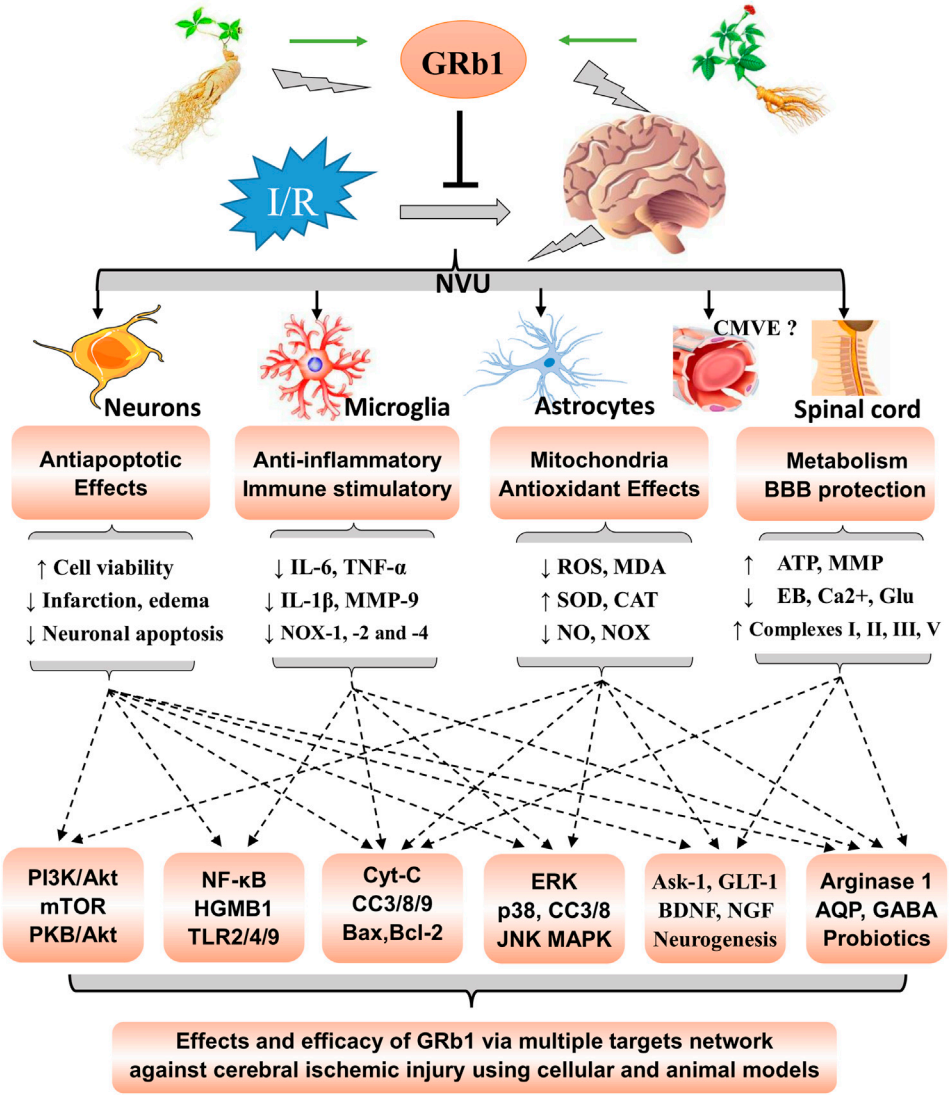

FIGURE 2 | Summarized effects and molecular network analysis of GRb1 in cerebral ischemic injury. Rb1 exerts significant neuroprotective effects on the neurovascular unit (NVU) and other neural cells *via* the network actions of its antiapoptotic, antioxidant, and anti-inflammatory activities; mitochondrial homeostasis; neurogenesis promotion; and regulation of the probiotic balance. The molecular mechanisms involve multiple effects, multiple targets, and multiple pathways. NVU, neurovascular unit; CMVE, cerebral microvascular endothelium. GRb1, ginsenoside Rb1; I/R, indicates ischemia and reperfusion. CC, cleaved caspase.

to 0.8–3.2 mg/kg in human (calculated from dose in mice) or 48–192 mg Rb1 for a 60 kg person. Thus, an effective concentration of Rb1 may be achievable in human.

CONCLUSION AND REMARKS

GRb1, a natural active ingredient, exerts neurotrophic and neuroprotective effects on the CNS. In the present review (**Figure 2**), we summarized the available reports on the therapeutic effects and the molecular mechanisms of GRb1 in cerebral I/R injury. The currently available data and our summarized results suggest that GRb1 may alleviate cerebral/neural ischemic injury *via* multiple pharmacological activities, such as its antiapoptotic, antioxidant, and anti-inflammatory properties, preservation of mitochondrial homeostasis, promotion of neurogenesis, and maintenance of the probiotic balance. Based on these results, GRb1, a special natural compound, has bright prospects for the prevention and treatment for ischemic stroke with a pharmacological network of multiple effects, targets, and molecular pathways.

Cerebral I/R injury is a complicated pathological process (Turley et al., 2005; Eltzschig and Eckle, 2011; Chamorro et al., 2016) that includes various types of I/R damage to the neurovascular unit (NVU), which is composed of nerve cells, BBB, microglia, and extracellular matrix (Lok et al., 2007; Guo and Lo, 2009; Lo, 2017). For many decades, neuronal injury has been considered the main cause of functional deficits after brain injury. Accordingly, almost all therapeutic strategies were

targeted at rescuing neurons and repairing neuronal damage. However, emerging data from both experimental models and patients now clearly show that, in patients with stroke, saving neurons alone may also be insufficient for treating brain infarcts (Lok et al., 2015) as brain functional connections and interactions among the different components in the NVU are also important (Cai et al., 2017; Jiang et al., 2018). Coincidentally, as shown in **Figure 2**, GRb1 exerts significant antiapoptotic effects on neurons exposed to cerebral ischemia stroke, suppresses neuroinflammation and microglia-mediated inflammatory stress in the acute phase of I/R, and protects ischemia-exposed astrocyte cells. Meanwhile, GRb1 inhibits oxidative stress and mitochondrial damage in cells exposed to I/R, preserves the BBB integrity, improves energy metabolism, and promotes neurogenesis. Moreover, GRb1 may alleviate spinal cord ischemia–reperfusion injury (SCII) and regulate the probiotic balance (**Figure 2**). Hence, GRb1 obviously plays a vital role not only in neural protection but also *via* the inhibition of NVU damage caused by CIRI.

From the perspective of protecting the NVU, GRb1 prevents I/R-induced cell apoptosis and death, suppresses excessive Ca^{2+}, glutamate, and RNS levels, alleviates mitochondrial stress, improves neurogenesis, and reduces the cerebral infarct volume *in vitro* and *in vivo*, indicating that GRb1 may exert antiapoptotic effects and protect the NVU from CIRI (**Figure 2**). Although the mechanisms have not been completely elaborated, our summarized results (**Table 1**) further reveal the molecular mechanisms and vital biomarkers of the effects of GRb1 on CIRI, which may be related to the PI3K-AKT-mTOR, autophagy, and mitochondrial apoptosis signaling pathways, NMDA receptor and GLT-1 targets, and neurogenesis.

Neuroinflammation underlies the etiology of multiple neurodegenerative diseases and stroke (Eldahshan et al., 2019). In the acute phase of ischemic stroke, resident microglia and recruited macrophages assume an M2 phenotype, and the immune-mediated inflammatory response is activated, resulting in the secretion of a number of inflammatory cytokines (IL-6, IL-1β, and TNF-α and the proteolytic enzymes MMP 3 and 9) (Jiang et al., 2018). Proinflammatory cytokines further trigger severe inflammatory stress, aggravate BBB dysfunction (Jiang et al., 2018), and induce NVU damage. All these events lead to the exacerbation of ischemic injury. Based on the summarized results listed in **Table 2**, we found that GRb1 inhibited the NF-KB, HMGB-1/TLR4, and HMGB1 signaling pathways, regulated the MAPK signaling pathway, increased the expression of GABA receptors, and reduced the levels of proinflammatory cytokines (IL-6, MMP-9, IL-1β, and TNF-α). By regulating these pathways, GRb1 suppresses neuroinflammation and microglia-mediated inflammatory reactions, reduces the damage caused by inflammatory factors, improves the integrity and normal functions of the BBB, and thus inhibits necrosis and apoptosis associated with anti-inflammatory activities at the early stages after stroke, which plays a fundamental role in the maintenance of CNS homeostasis, BBB structural integrity, and normal functions of the NVU.

The neutralization of oxidative and nitrosative stresses is a potential therapeutic strategy because the ischemic brain is highly susceptible to oxidative damage (Crack and Taylor, 2005; Xie et al., 2020). After CI/RI, the production of ROS increases, leading to lipid peroxidation, mitochondrial and DNA damage, protein nitration, and mitochondrial injury that evokes the mitochondrial release of apoptosis inducers (Bolaños et al., 2009; Chamorro et al., 2016). According to recent studies (**Table 3**), GRb1 decreases the production of ROS, MDA, and NO, improves antioxidant defenses (CAT, SOD, and GSH-px), and inhibits I/R-induced mitochondrial injury *in vivo* and *in vitro*. Moreover, it remarkably increases AQP4 levels (Li et al., 2019), decreases Bax expression (Yang et al., 2008), upregulates the expression of Bcl-2 (Yang et al., 2008), downregulates NOX-4 expression and NOX activities, and inhibits the cascade reactions of caspase-3 and caspase-9. Thus, GRb1 exerts significant neuroprotective effects due to its antioxidant activity and protects the mitochondria in the treatment of cerebral ischemic injury.

In summary, this review addresses the effects and efficacy of GRb1 by discussing research results obtained using cellular and animal models; the summarized results and analysis indicate that GRb1, a new candidate agent, has bright prospects for preventing and treating ischemic stroke.

However, unrecognized actions and limitations of GRb1 still exist. 1) Researchers have not clearly determined whether GRb1 alleviates ischemic injury of the cerebral microvascular endothelium (CMVE) of the NVU. 2) Because of the lack of clinical testing and validation of preclinical data, the use of GRb1 as a new drug for treating ischemic stroke remains a challenge. 3) The existing research and data only showed the effects of GRb1 on I/R in the early stages and acute phases. Therefore, currently available clinical trials and data collection are urgently needed, and future studies should focus on the effects and molecular mechanisms of GRb1 on the NVU system and the recovery phase of ischemic stroke.

AUTHOR CONTRIBUTIONS

WX and XW designed the review; WX, XW, and YW wrote the manuscript; YC, DY, and TX helped map the figures and revise the manuscript. SZ was mainly responsible for the supervision of the works and the management of related projects. All authors discussed, edited and approved the final version.

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SYSTEMATIC REVIEW
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Herbal Medicine for Behavioral and Psychological Symptoms of Dementia: A Systematic Review and Meta-Analysis

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Background: Dementia is a global health concern, causing serious health and socioeconomic burdens with population aging. The associated symptoms of dementia, called behavioral and psychological symptoms of dementia (BPSD), are factors contributing to the socioeconomic burden of dementia. Recently, herbal medicine (HM) has attracted attention as a potential complementary therapy for BPSD. Therefore, this systematic review was aimed at analyzing the effectiveness (or efficacy), safety, and research status of HM in BPSD management through a comprehensive review.

Methods: Thirteen electronic databases were searched comprehensively. Related clinical studies published until December 28, 2020, were collected. The methodological quality was evaluated using tools such as the Cochrane Collaboration's risk of bias tool according to the study design. The effectiveness (or efficacy) was analyzed for randomized controlled trials (RCTs) only, and when sufficient homogeneity was assured, effect estimates were presented as mean difference (MD) and risk ratio (RR), with 95% confidence interval (CIs), through a meta-analysis.

Results: A total of 52 clinical studies, including 36 RCTs, were included in this review. As an adjunctive therapy, HM showed statistically significant benefits in BPSD severity assessed by the Behavior Pathology in Alzheimer's Disease Rating Scale (combined with psychotropic drugs: MD = −3.48, 95% CI: −3.96 to −2.99; with anti-dementia drugs: MD = −2.81, 95% CI: −3.17 to −2.45) and Neuropsychiatric Inventory (with anti-dementia drugs: MD = −3.23, 95% CI: −4.06 to −2.40). Adverse events were significantly less frequent in the HM group (RR = 0.50; 95% CI: 0.28 to 0.88). However, the methodological quality of the RCTs included in this systematic review was not optimal overall.

Conclusion: According to the findings of this review, HM may be associated with additional benefits in BPSD treatment, particularly when used as an adjunct to conventional medications, including psychotropic and anti-dementia drugs. However, considering the methodological quality of the included RCTs, this clinical evidence is not robust. Nevertheless, dementia is a global health concern, and considering the limitations of conventional psychotropic drugs for BPSD, a major cause of the disease burden, HM appears to be a promising complementary therapy that warrants further research.

Keywords: dementia, BPSD, EATM, herbal medicine, systematic review

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INTRODUCTION

Dementia is a global health concern, causing serious health and socioeconomic burdens with population aging. A study comparing its prevalence and costs between 2010 and 2015 calculated overall annual trends and predicted that the worldwide costs of dementia in 2030 would reach approximately US $2 trillion (Wimo et al., 2017). The clinical manifestation of dementia can be classified into cognitive decline, i.e., core symptoms, and associated symptoms called behavioral and psychological symptoms of dementia (BPSD) (Ohno et al., 2019). BPSD is a term that encompasses various behavioral problems and psychological symptoms that may occur in patients with dementia and is related to the poor patient prognosis, burden of caregivers, and risk of institutionalization, consequently contributing to the socioeconomic burden of dementia (Cerejeira et al., 2012). BPSD is present in most patients with dementia, particularly hyperactivity, apathy, depression, and anxiety, with moderate or higher incidence (van der Linde et al., 2016).

Although pharmacological approaches, including psychotropic drugs, are frequently used to manage BPSD in clinical settings (Ozaki et al., 2017), the results are occasionally unsatisfactory, and drugs such as antipsychotics, benzodiazepines, and Z-drugs are associated with adverse events (AEs), such as increased risk of falls and all-cause mortality (Landi et al., 2005; Ralph and Espinet, 2018). Moreover, patients with dementia are mostly elderly, and the use of several psychotropic drugs in the population is considered a "potentially inappropriate medication," which discourages the use of psychotropic drugs for BPSD (By the 2019 American Geri, 2019). Therefore, more effective and safe treatments for BPSD management are necessary.

East Asian traditional medicine (EATM) is a medical system that has been established in Asian countries for a long time, and some countries, such as Korea, Japan, China, and Taiwan, use it in their national medical systems (Park et al., 2012). As an EATM modality, herbal medicine (HM) is considered to be a management strategy for dementia, particularly BPSD. For example, an HM called Yokukansan is effective against the positive symptoms of BPSD (Matsuda et al., 2013). However, other types of HMs can also be considered in the management of BPSD, highlighting the need for a comprehensive review of the various HMs that can be used in BPSD (Howes et al., 2017). Therefore, this systematic review was aimed at analyzing the effectiveness (or efficacy), safety, and research status of HM in BPSD management through a comprehensive review.

MATERIALS AND METHODS

We registered the protocol of this systematic review in the OSF registries (URL: https://osf.io/3u8ch) and International Prospective Register of Systematic Reviews (URL: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42020211000) before beginning the study. The study protocol was as previously described (Kwon et al., 2021). No amendments were made to the information provided in the protocol. We report the systematic review in accordance with the Preferred Reporting Items for Systematic Review and Meta-Analysis 2020 checklist (Page et al., 2021).

Information Sources and Search Strategy

One researcher (B Lee) searched MEDLINE *via* PubMed, EMBASE *via* Elsevier, the Cochrane Central Register of Controlled Trials, Allied and Complementary Medicine Database *via* EBSCO, Cumulative Index to Nursing and Allied Health Literature *via* EBSCO, PsycARTICLES *via* ProQuest, Oriental Medicine Advanced Searching Integrated System, Koreanstudies Information Service System, Research Information Service System, Korean Medical Database, Korea Citation Index, China National Knowledge Infrastructure, and Wanfang Data on December 28, 2020. Articles published from the inception of the database to the search date were screened. We also identified additional eligible articles through reviews of relevant literature reference lists and trial registries, such as clinicaltrials.gov, and consultation with experts in this area to include additional gray literature. The detailed search strategies are described in **Supplementary Material S1**.

Eligibility Criteria

We included all types of original clinical studies, including randomized controlled clinical trials (RCTs), non-randomized controlled clinical trials (CCTs), and before–after studies without restrictions on the publication language or publication status. Studies involving patients with any type of dementia in long-term care facilities, community, or specialized geriatric assessments and psychiatric units were included. Although there were no restrictions on the sex, age, or race of the participants, studies that did not provide diagnostic criteria or a validated assessment tool for inclusion and studies on patients with drug allergies or other serious illnesses, such as cancer, liver disease, or kidney disease, were excluded. We included studies involving oral HM based on EATM theories as a monotherapy or adjunctive therapies to psychotropic drugs, with or without routine care for dementia as treatment interventions. Although there were no restrictions on the dosage form of HM, we excluded studies that did not list the composition of HM, except for patent drugs. For the control intervention, we included studies involving wait-list, placebo, or psychotropic drugs, with or without routine care for dementia, such as anti-dementia drugs.

The primary outcome was the severity of BPSD symptoms, such as scores of the Behavior Pathology in Alzheimer's Disease Rating Scale (BEHAVE-AD) (Sclan et al., 1996), Neuropsychiatric Inventory (NPI) (Cummings et al., 1994), and Brief Psychiatric Rating Scale (BPRS) (Overall and Gorham, 1962). The secondary outcomes included 1) total effective rate (TER) for BPSD symptoms; 2) activities of daily living (ADLs) of patients, such as the Barthel Index (Mahoney and Barthel, 1965) and the Functional Independence Measure

(Linacre et al., 1994), as well as instrumental ADL (IADL), such as the Activities of Daily Living Prevention Instrument (Galasko et al., 2006); 3) quality of life (QoL) of patients, such as the Alzheimer Disease Related Quality of Life (Kasper et al., 2009); 4) caregiver burden of caregivers, such as the Caregiver Burden Inventory (Novak and Guest, 1989); 5) QoL of caregivers, such as the Short Form 36 Health Survey (Ware and Sherbourne, 1992); 6) placement in a long-term care facility from home; and 7) safety data, such as incidence of AEs.

Study Selection

All documents retrieved from the databases and other sources were imported into EndNote X8 (Clarivate Analytics, Philadelphia, United States). Using "Find Duplicates" function in EndNote X8 and manual searching, duplicate documents were excluded, and two researchers (CY Kwon and B Lee) independently reviewed the possibility of inclusion by reviewing the titles and abstracts. For the first included documents, the final documents to be included were determined through a review of full texts. Disagreements between the two researchers in the study selection process were resolved through consensus.

Data Extraction

Two researchers (CY Kwon and B Lee) independently extracted the data from the included studies using a pre-defined form in Excel 2016 (Microsoft, Redmond, WA, United States). The extracted information included the first author's name, publication year, country, sample size and dropout, details of participants, treatment and control intervention, duration of intervention, main outcome measures and results after treatment ended, AEs, and information to assess the risk of bias (RoB). When the data in each included study were insufficient, we contacted the corresponding authors of the original studies *via* e-mail. Disagreements between the researchers in the data extraction process were resolved through consensus.

RoB Assessment

To assess the RoB of the included RCTs, we used Cochrane Collaboration's RoB tool comprising domains of random sequence generation, allocation concealment, blinding of participants, personnel, and outcome assessors, completeness of outcome data, selective reporting, and other biases. In particular, we assessed other bias items based on the statistical baseline imbalance between the treatment and control groups, such as the participant's mean age, sex, disease period, or disease severity. Each domain was assessed as "low risk," "unclear risk," or "high risk" (Higgins, 2011), and the evaluation results are presented as a figure using Review Manager software, version 5.4 (Cochrane, London, United Kingdom). For included CCTs, before–after studies, and case reports, we used the Risk Of Bias In Non-randomized Studies of Interventions tool (Sterne et al., 2016), The Quality Assessment Tool for Before–After (Pre–Post) Studies With No Control Group National Heart, Lung, and Blood Institute (NHLBI) (2013), and the Quality Assessment Tool for Case Series Studies National Heart, Lung, and Blood Institute (NHLBI) (2013), respectively. Two researchers (CY Kwon and B Lee) independently assessed the RoB of the included studies, and discrepancies were resolved through consensus.

Data Synthesis and Analysis

A descriptive analysis of the findings, including the demographic characteristics of the participants, details of the interventions, and outcomes, were conducted for all included studies. If there were two or more studies using the same type of treatment and control interventions, with the same outcome measures among our primary and secondary outcomes, a meta-analysis was conducted using Review Manager software (version 5.4; Cochrane, London, United Kingdom). For continuous and binary outcomes, the mean difference (MD) and risk ratio (RR) were calculated with 95% confidence interval (CI). We assessed heterogeneity using both the χ^2 test and the I^2 statistic, and I^2 values greater than 50 and 75% were interpreted as substantial and considerable heterogeneity, respectively. We pooled the meta-analyzed results using a random-effects model if the included studies had significant heterogeneity ($I^2 > 50\%$) and a fixed-effect model if the heterogeneity was insignificant or if less than five studies were included in the meta-analysis because of lack of precision in the estimate of the between-study variance (Guyatt et al., 2002; Balshem et al., 2011). We planned subgroup analyses according to the severity of dementia, type of dementia, severity of BPSD, and treatment duration, if necessary data were available. The Mini-Mental State Examination score was used to classify the severity of dementia of the participants, with scores of 20–24, 13–20, and 12 or less regarded as mild, moderate, and severe, respectively. Additionally, we conducted a sensitivity analysis to identify the robustness of the results of the meta-analysis by excluding 1) studies with high RoB and 2) outliers that are numerically distant from the rest of the data. If more than ten studies were included in each meta-analysis, we planned to assess the publication bias using a funnel plot.

RESULTS

Study Selection

A total of 21,673 articles were identified through the database search, and there were no additional records from other sources. After removing duplicates, the titles and abstracts of 16,560 articles were screened for inclusion. After excluding 16,415 articles, the full texts of the remaining 145 articles were assessed for final inclusion. We excluded a total of 93 articles, including one for not being an original article, six for not being clinical studies, 11 for being only abstracts without raw data, two for not being about dementia, three for having accompanying diseases other than dementia, eight for not reporting diagnostic criteria of dementia, three for not being about oral HM, six for not reporting details of HM, two for comparing different HMs, three for using traditional Chinese medicine other than HM, 36 for not reporting the outcome of interest, eight for using duplicate data,

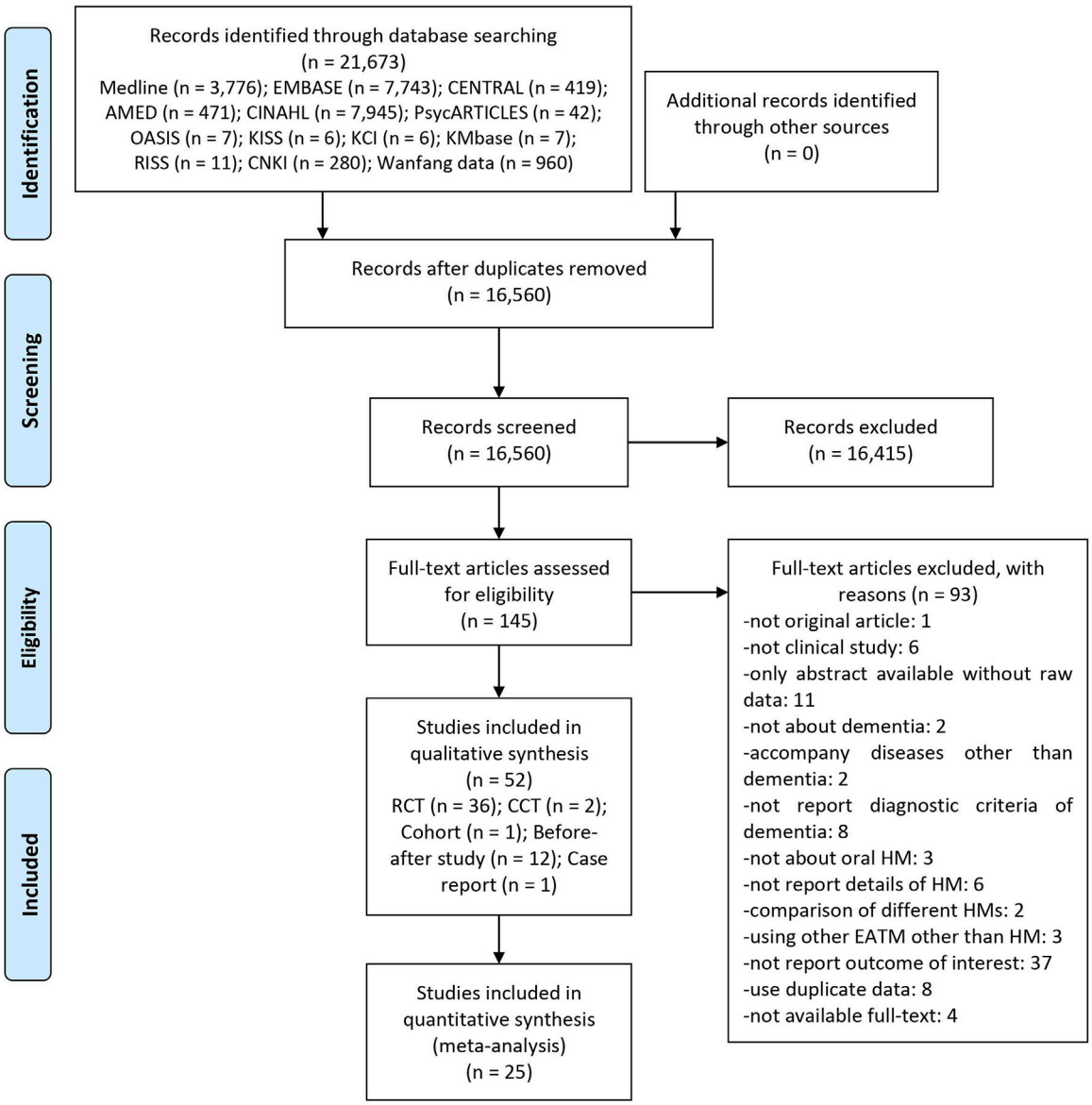

FIGURE 1 | A PRISMA flow diagram of the literature screening and selection processes. AMED, Allied and Complementary Medicine Database; CENTRAL, Cochrane Central Register of Controlled Trials; CCT, non-randomized controlled clinical trial; CINAHL, Cumulative Index to Nursing and Allied Health Literature; CNKI, China National Knowledge Infrastructure; HM, herbal medicine; KCI, Korea Citation Index; KISS, Koreanstudies Information Service System; KMbase, Korean Medical Database; OASIS, Oriental Medicine Advanced Searching Integrated System; RCT, randomized controlled trial; RISS, Research Information Service System; TCM, traditional Chinese medicine.

and four for unavailable full-texts (**Supplementary Material S2**). Finally, we reviewed 52 studies, including 36 RCTs (Chen et al., 1997; Terasawa et al., 1997; Motohashi, 2006; Mizukami et al., 2009; Monji et al., 2009; Okahara et al., 2010; Guo et al., 2011a; Zhang, 2012; Chen et al., 2013; Shen, 2013; Teranishi et al., 2013; Pan et al., 2014; Pu et al., 2014; Yao et al., 2014; Zhang et al., 2015a; Du et al., 2015; Hu et al., 2015; Liu et al., 2015; Zhou, 2015; Zhou and Wei, 2015; Lin et al., 2016; Furukawa et al., 2017; Zuo, 2017; Fang et al., 2018; Gu et al., 2018; Han, 2018; Li et al., 2018; Shen et al., 2018; Zhang et al., 2018; Zhou, 2018; Huang and Xu, 2019; Shen et al., 2019; Zhu et al., 2019; Chen, 2020; Li, 2020; Shi et al., 2020), two CCTs (Kudoh et al., 2016; Xu, 2018), one cohort (Meguro and Yamaguchi, 2018), 12 before–after studies (Iwasaki et al., 2005; Xu et al., 2007; Shinno et al., 2008; Hayashi et al., 2010; Kawanabe et al., 2010; Guo et al., 2011b; Iwasaki et al., 2012; Nagata et al., 2012; Yang et al., 2012; Sumiyoshi et al., 2013; Ohsawa et al., 2017; Manabe, 2020), and one case report (Shinno et al., 2007). Among them, 25 RCTs (Terasawa et al., 1997; Monji et al., 2009; Guo et al., 2011a; Zhang, 2012; Chen et al., 2013; Shen, 2013; Pan et al., 2014; Pu et al., 2014; Zhang et al., 2015a; Du et al., 2015; Liu et al., 2015; Zhou, 2015; Zhou and Wei, 2015; Lin et al., 2016; Furukawa et al., 2017; Zuo, 2017; Fang et al., 2018; Gu et al.,

2018; Han, 2018; Li et al., 2018; Shen et al., 2018; Huang and Xu, 2019; Zhu et al., 2019; Li, 2020; Shi et al., 2020) were included in the meta-analysis (**Figure 1**).

Study Characteristics

Thirty-four studies (Chen et al., 1997; Chen et al., 2013; Chen, 2020; Du et al., 2015; Fang et al., 2018; Gu et al., 2018; Guo et al., 2011a; Han, 2018; Hu et al., 2015; Huang and Xu, 2019; Li, 2020; Li et al., 2018; Lin et al., 2016; Liu et al., 2015; Motohashi, 2006; Pan et al., 2014; Pu et al., 2014; Shen, 2013; Shen et al., 2019; Shen et al., 2018; Shi et al., 2020; Yao et al., 2014; Zhang et al., 2015a; Zhang et al., 2018; Zhang, 2012; Zhou, 2018; Zhou, 2015; Zhou and Wei, 2015; Zhu et al., 2019; Zuo, 2017; Xu, 2018; Guo et al., 2011b; Xu et al., 2007; Yang et al., 2012) were published in China, and 18 studies (Furukawa et al., 2017; Mizukami et al., 2009; Monji et al., 2009; Okahara et al., 2010; Teranishi et al., 2013; Terasawa et al., 1997; Kudoh et al., 2016; Meguro and Yamaguchi, 2018; Hayashi et al., 2010; Iwasaki et al., 2012; Iwasaki et al., 2005; Kawanabe et al., 2010; Manabe, 2020; Nagata et al., 2012; Ohsawa et al., 2017; Shinno et al., 2008; Sumiyoshi et al., 2013; Shinno et al., 2007) were published in Japan. The type of dementia was Alzheimer's disease, vascular dementia, two or more types of dementia, and dementia with Lewy bodies in 26 (Chen, 2020; Fang et al., 2018; Furukawa et al., 2017; Gu et al., 2018; Guo et al., 2011a; Han, 2018; Hu et al., 2015; Li, 2020; Li et al., 2018; Lin et al., 2016; Liu et al., 2015; Monji et al., 2009; Okahara et al., 2010; Pan et al., 2014; Zhang et al., 2015a; Zhang et al., 2018; Zhou, 2018; Zhou, 2015; Zhou and Wei, 2015; Zuo, 2017; Kudoh et al., 2016; Meguro and Yamaguchi, 2018; Guo et al., 2011b; Hayashi et al., 2010; Ohsawa et al., 2017; Yang et al., 2012), 12 (Chen et al., 1997; Motohashi, 2006; Pu et al., 2014; Shen et al., 2019; Shen et al., 2018; Shi et al., 2020; Terasawa et al., 1997; Yao et al., 2014; Zhu et al., 2019; Xu, 2018; Nagata et al., 2012; Xu et al., 2007), five (Chen et al., 2013; Du et al., 2015; Shen, 2013; Zhang, 2012; Sumiyoshi et al., 2013), and four (Iwasaki et al., 2012; Iwasaki et al., 2005; Manabe, 2020; Shinno et al., 2007) studies, respectively. Among RCTs, HM was evaluated as a monotherapy, control, psychotropic drug, and placebo in 16 (Chen et al., 1997; Chen et al., 2013; Furukawa et al., 2017; Mizukami et al., 2009; Pan et al., 2014; Pu et al., 2014; Shen, 2013; Shi et al., 2020; Teranishi et al., 2013; Terasawa et al., 1997; Zhang et al., 2015a; Zhang, 2012; Zhou, 2018; Zhou, 2015; Zhou and Wei, 2015; Zuo, 2017), nine (Chen et al., 2013; Pu et al., 2014; Shen, 2013; Teranishi et al., 2013; Zhang et al., 2015a; Zhang, 2012; Zhou, 2018; Zhou, 2015; Zuo, 2017), and four (Furukawa et al., 2017; Pan et al., 2014; Shi et al., 2020; Terasawa et al., 1997) studies, respectively. Twenty studies (Chen, 2020; Du et al., 2015; Fang et al., 2018; Gu et al., 2018; Guo et al., 2011a; Han, 2018; Hu et al., 2015; Huang and Xu, 2019; Li, 2020; Li et al., 2018; Lin et al., 2016; Liu et al., 2015; Monji et al., 2009; Motohashi, 2006; Okahara et al., 2010; Shen et al., 2019; Shen et al., 2018; Yao et al., 2014; Zhang et al., 2018; Zhu et al., 2019) evaluated HM as an adjunctive therapy. Anti-dementia drugs were the most used as a control group in 11 studies (Chen, 2020; Du et al., 2015; Fang et al., 2018; Guo et al., 2011a; Han, 2018; Hu et al., 2015; Li, 2020; Liu et al., 2015; Okahara et al., 2010; Shen et al., 2018; Zhu et al., 2019), followed by psychotropic drugs in six studies (Gu et al., 2018; Huang and Xu, 2019; Li et al., 2018; Lin et al., 2016; Monji et al., 2009; Zhang et al., 2018). In a total of 13 studies (Chen et al., 2013; Guo et al., 2011a; Han, 2018; Hu et al., 2015; Huang and Xu, 2019; Lin et al., 2016; Pu et al., 2014; Yao et al., 2014; Zhang et al., 2018; Zhou, 2015; Zhu et al., 2019; Guo et al., 2011b; Yang et al., 2012), participants were recruited according to pattern identification, of which blood stasis was the most common (six studies) (Chen et al., 2013; Hu et al., 2015; Pu et al., 2014; Yao et al., 2014; Zhu et al., 2019; Yang et al., 2012), followed by phlegm (five studies) (Chen et al., 2013; Hu et al., 2015; Lin et al., 2016; Zhu et al., 2019; Yang et al., 2012) or kidney deficiency (five studies) (Chen et al., 2013; Guo et al., 2011a; Hu et al., 2015; Huang and Xu, 2019; Guo et al., 2011b). The treatment period ranged from 2 weeks to 2 years, of which 4 weeks (1 month) was the most common in 20 studies (Chen et al., 2013; Chen, 2020; Furukawa et al., 2017; Guo et al., 2011a; Li, 2020; Mizukami et al., 2009; Okahara et al., 2010; Zhou, 2018; Xu, 2018; Guo et al., 2011b; Hayashi et al., 2010; Iwasaki et al., 2012; Iwasaki et al., 2005; Kawanabe et al., 2010; Manabe, 2020; Nagata et al., 2012; Shinno et al., 2008; Sumiyoshi et al., 2013; Yang et al., 2012; Shinno et al., 2007), followed by 8 weeks (2 months) in 12 studies (Chen et al., 1997; Fang et al., 2018; Huang and Xu, 2019; Lin et al., 2016; Motohashi, 2006; Pu et al., 2014; Shen et al., 2019; Shen et al., 2018; Teranishi et al., 2013; Zhang, 2012; Zhu et al., 2019; Zuo, 2017) and 12 weeks (3 months) in 10 studies (Du et al., 2015; Gu et al., 2018; Han, 2018; Li et al., 2018; Monji et al., 2009; Shen, 2013; Terasawa et al., 1997; Yao et al., 2014; Zhou and Wei, 2015; Ohsawa et al., 2017). After completion of treatment, the follow-up was performed in four studies (Fang et al., 2018; Pan et al., 2014; Zhang et al., 2015a; Kawanabe et al., 2010), of which the duration was 4 weeks in two studies (Fang et al., 2018; Kawanabe et al., 2010) and 5 (Pan et al., 2014) and 24 weeks (Zhang et al., 2015a) in one study each. Eighteen studies (Furukawa et al., 2017; Lin et al., 2016; Mizukami et al., 2009; Monji et al., 2009; Okahara et al., 2010; Pan et al., 2014; Shi et al., 2020; Teranishi et al., 2013; Zhang et al., 2015a; Kudoh et al., 2016; Meguro and Yamaguchi, 2018; Hayashi et al., 2010; Iwasaki et al., 2012; Manabe, 2020; Nagata et al., 2012; Ohsawa et al., 2017; Shinno et al., 2008; Sumiyoshi et al., 2013) were approved by the institutional review board before the study began, and 37 studies (Chen, 2020; Du et al., 2015; Furukawa et al., 2017; Gu et al., 2018; Han, 2018; Hu et al., 2015; Huang and Xu, 2019; Li, 2020; Lin et al., 2016; Liu et al., 2015; Mizukami et al., 2009; Monji et al., 2009; Okahara et al., 2010; Pan et al., 2014; Pu et al., 2014; Shen, 2013; Shen et al., 2019; Shen et al., 2018; Shi et al., 2020; Teranishi et al., 2013; Terasawa et al., 1997; Zhang et al., 2015a; Zhang et al., 2018; Zhou, 2018; Zhou, 2015; Zhu et al., 2019; Zuo, 2017; Kudoh et al., 2016; Meguro and Yamaguchi, 2018; Hayashi et al., 2010; Iwasaki et al., 2012; Iwasaki et al., 2005; Manabe, 2020; Nagata et al., 2012; Ohsawa et al., 2017; Shinno et al., 2008; Sumiyoshi et al., 2013) received consent forms from participants (**Table 1**, **Supplementary Material S3**). Various types of HMs were used in the included studies, of which Yokukansan was the most frequently used in 13 studies (Iwasaki et al., 2005; Shinno et al., 2007; Shinno et al., 2008; Mizukami et al., 2009; Monji et al., 2009; Hayashi et al., 2010; Kawanabe et al., 2010; Okahara et al., 2010; Iwasaki et al., 2012; Nagata et al., 2012; Sumiyoshi

TABLE 1 | Basic characteristics of the randomized controlled trial.

Study ID	Sample size (included →analyzed)	Mean age (year)	Sex (M:F)	Population (Diagnosis)	Pattern identification	(A) Treatment intervention	(B) Control intervention	Treatment duration/ Follow-up	Outcome
Chen et al. (1997)	61 (32:29) →61(32:29)	(A) 64.32 ± 5.42 (B) 63.21 ± 6.41	(A) 32(27:5) (B) 29(21:8)	-VD (DSM-IV, ICD-10) -MMSE≤23 (A) 19.0 ± 3.4 (B) 18.6 ± 4.5 -HIS<24 -HAMD (A) 18.0 ± 6.4 (B) 17.5 ± 6.3	NA	HM	Hydergine 6 mg/day	2 months/ NR	1. MMSE 2. HAMD 3. TER (TCM symptom score) 4. TER (Neurological deficit) 5. TER (ADL) 6. TER (MMSE) 7. Gait and balance function 8. Cerebral blood flow 9. TER (Electroencephalography)
Chen et al. (2013)	60(30:30) →60(30:30)	(A) 73.3 ± 5.1 (B) 74.3 ± 7.4	(A) 30(20:10) (B) 30(23:7)	-AD or VD (CCMD-3, ICD-10) -MMSE≤24 -BEHAVE-AD≥8 (A) 16.3 ± 7.3 (B) 15.8 ± 6.9	Sea of marrow deficiency, dual deficiency of spleen-kidney, liver-kidney deficiency, phlegm turbidity obstructing the orifices, blood stasis due to qi stagnation	HM	Risperidone 0.5 mg/ day (modification up to 3 mg/day, according to patient's condition)	4 weeks/NR	1. TER (BEHAVE-AD) 2. BEHAVE-AD
Chen (2020)	80(40:40) →80(40:40)	(A) 74.52 ± 2.65 (B) 73.82 ± 2.88	(A) 40(22:18) (B) 40(24:16)	-AD -Insomnia (DSM-5) -PSQI>7	NA	HM + (B)	Health education, donepezil 5 mg/day	1 month/NR	1. PSQI
Du et al. (2015)	105(51:54) →105(51:54)	(A) 74.9 (B) 74.7	(A) 51(31:20) (B) 54(32:22)	-AD or VD (DSM-IV) -BEHAVE-AD≥8 (A) 18.3 ± 3.9 (B) 18.7 ± 4.0 -GDS<5	NA	HM + (B)	Memantine 5 mg/day (1 week: 5 mg/day, 2 weeks: 10 mg/day, 3 weeks: 15 mg/day, 4 weeks: 20 mg/day)	3 months/NR	1. TER (BEHAVE-AD) 2. BEHAVE-AD 3. ACE-R 4. Barthel index
Fang et al. (2018)	90(45:45) →90(45:45)	(A) 71.5 ± 9.1 (B) 70.4 ± 8.4	(A) 45(26:19) (B) 45(25:20)	-AD+depression (DSM-IV) -MoCA (A) 14.44 ± 3.14 (B) 14.82 ± 3.25 -HAMD (A) 22.53 ± 3.15 (B) 23.11 ± 3.25	NA	HM + (B)	Donepezil 5 mg/day (10 mg/day after 4 weeks)	8 weeks/ 4 weeks	1. MoCA 2. HAMD 3. Serum 5-HT 4. Serum dopamine
Furukawa et al. (2017)	145(75:70) →129(65:64)	(A) 78.3 ± 5.4 (B) 78.5 ± 5.1	(A) 75(33:42) (B) 70(28:42)	-AD (NINCDS-ADRDA) -NPI-Q>4, agitation/aggression + irritability/lability>2 (A) 9.6 ± 4.2 (B) 9.4 ± 4.4 -MMSE 10-26 (A) 19.7 ± 3.9 (B) 19.0 ± 4.4	NA	HM	Placebo	4 weeks/NR	1. NPI-Q 2. MMSE

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TABLE 1 | (*Continued*) Basic characteristics of the randomized controlled trial.

Study ID	Sample size (included →analyzed)	Mean age (year)	Sex (M:F)	Population (Diagnosis)	Pattern identification	(A) Treatment intervention	(B) Control intervention	Treatment duration/ Follow-up	Outcome
Gu et al. (2018)	80(40:40) →80(40:40)	(A) 66.8 ± 7.6 (B) 71.7 ± 6.4	(A) 40(21:19) (B) 40(18:22)	-AD (CCMD) -BPRS≥35 (A) 63 ± 3 (B) 61 ± 7	NA	HM + (B)	Olanzapine 2.5 mg/day (modification according to patient's condition)	3 months/ NR	1. TER (BPRS symptom) 2. BPRS
Guo et al. (2011a)	60(30:30) →60(30:30)	(A) 73.8 ± 1.02 (B) 73.14 ± 0.96	(A) 30(16:14) (B) 30(18:12)	-AD (NINCDS-ADRDA) -BEHAVE-AD≥8 (A) 13.77 ± 2.66 (B) 13.57 ± 2.77	Liver-kidney deficiency	HM + (B)	Donepezil 5 mg/day (modification according to patient's condition)	1 month/NR	1. BEHAVE-AD 2. TER (BEHAVE-AD) 3. MMSE
Han (2018)	47 (29:28) →47(29:28)	(A) 61.35 ± 6.28 (B) 62.38 ± 6.15	(A) 29(18:11) (B) 28(16:12)	-AD (CCMD-3) -MMSE 12-24 (A) 18.5 ± 2.7 (B) 18.5 ± 2.8 -ADAS-cog (A) 11.6 ± 2.6 (B) 12.5 ± 2.4	qi blood deficiency	HM + (B)	Rivastigmine 3 mg/day	3 months/ NR	1. MMSE 2. ADAS-cog 3. Bathel index 4. NPI 5. SDSD 6. TER (TCM symptom score)
Hu et al. (2015)	80(40:40) →80(40:40)	(A) 68.4 ± 7.2 (B) 69.2 ± 6.4	(A) 40(26:14) (B) 40(25:15)	-AD (NINCDS-ADRDA, DSM-IV) -MMSE 21-26 (A) 15.28 ± 2.74 (B) 15.49 ± 2.87 -ADAS-cog (A) 65.71 ± 7.95 (B) 64.27 ± 7.36	Kidney essence deficiency, phlegm and stasis obstruction	HM + (B)	Donepezil 10 mg/day, Piracetam 2.4g/day	6 months/ NR	1. TER (clinical symptom) 2. MMSE 3. ADAS-cog 4. ADL 5. NPI-Q 6. Serum SOD 7. Serum MDA 8. Serum TNF-α 9. Serum IL-1 10. Serum IL-6
Huang and Xu (2019)	90(45:45) →90(45:45)	(A) 69.6 ± 5.1 (B) 67.7 ± 4.7	(A) 45(23:22) (B) 45(24:21)	-SD (ICD, DSM) -MMSE<17 (A) 13.3 ± 3.2 (B) 13.8 ± 3.1 -HDS<16 (A) 12.7 ± 3.2 (B) 12.6 ± 3.1 -BEHAVE-AD>8 -ADL≥22	Liver-kidney deficiency, dual deficiency of spleen-kidney, and sea of marrow deficiency	HM + (B)	Olanzapine 2.5 mg/ day (modification up to 20 mg/day, according to patient's condition)	2 months/ NR	1. BEHAVE-AD 2. HDS 3. MMSE 4. ADL 5. Serum SOD 6. Serum MDA 7. Serum IL-6 8. Serum IL-1 9. TER (BEHAVE-AD)
Li et al. (2018)	100(50:50) →100(50:50)	(A) 69.87 ± 2.65 (B) 68.19 ± 2.73	(A) 50(24:26) (B) 50(23:27)	-AD (Textbook in psychiatry for Asia) -Behavior disorder	NA	HM + (B)	Clonapine 25 mg/day (after 2–3 weeks, +20–50 mg/3–4 days up to 200 mg/day)	12 weeks/ NR	1. Serum SOD 2. Serum MDA 3. BEHAVE-AD 4. TER (BEHAVE-AD)
Li (2020)	82(41:41) →82(41:41)	(A) 69.35 ± 4.08 (B) 70.67 ± 3.78	(A) 41(23:18) (B) 41(25:16)	-AD [2018 Guidelines for the diagnosis and treatment of dementia and cognitive impairment in China(2)] -MMSE 10-25 (A) 17.32 ± 2.58 (B) 16.89 ± 2.58	NA	HM + (B)	Donepezil 5 mg/day	1 month/NR	1. TER (Positive and Negative Syndrome Scale, MMSE) 2. MMSE 3. BEHAVE-AD 4. Plasma Hcy 5. Plasma CRP

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TABLE 1 | (*Continued*) Basic characteristics of the randomized controlled trial.

Study ID	Sample size (included →analyzed)	Mean age (year)	Sex (M:F)	Population (Diagnosis)	Pattern identification	(A) Treatment intervention	(B) Control intervention	Treatment duration/ Follow-up	Outcome
Lin et al. (2016)	92(46:46) →84(41:43)	(A) 71.8 ± 6.4 (B) 70.5 ± 6.7	(A) 46(24:22) (B) 46(26:20)	-AD (CCMD-3) -MMSE≤24 (A) 15.18 ± 3.05 (B) 15.04 ± 3.27 -BEHAVE-AD≥8	non-interaction between the heart and kidney and phlegm turbidity	HM + (B)	Aripiprazole 2.5 mg/day	8 weeks/NR	1. MMSE 2. BEHAVE-AD 3. TER (BPRS)
Liu et al. (2015)	86(43:43) →86(43:43)	(A) 71 ± 4 (B) 72 ± 3	(A) 43(18:25) (B) 43(20:23)	-AD (Guidelines for the diagnosis and treatment of dementia and cognitive impairment in China) -MMSE 10-24 (A) 17.9 ± 3.0 (B) 16.9 ± 3.0 -CDR (A) 2.00 ± 0.30 (B) 1.90 ± 0.20	NA	HM + (B)	Donepezil 5 mg/day (modification up to 10 mg/day)	6 months/ NR	1. BEHAVE-AD 2. CDR 3. NPI 4. Plasma 8-isoprostane F2α 5. Urine 8-isoprostane F2α
Mizukami et al. (2009)	103(53:50) →103(53:50)	(A) outpatient 80.6 ± 3.9; inpatient 78.9 ± 6.9 (B) outpatient 76.9 ± 6.1; inpatient 78.0 ± 6.7	(A) 53(25:5) (B) 50(20:6)	-AD (NINCDS-ADRDA, DSM-IV) or DLB (Consensus guidelines for the clinical and pathologic diagnosis of DLB) -MMSE (A) outpatient 17.4 ± 6.3; inpatient 9.8 ± 6.9 (B) outpatient 14.9 ± 5.6; inpatient 9.4 ± 6.7 -NPI≥6 for at least one of ten items (A) outpatient 25.5 ± 12.0; inpatient 22.1 ± 13.2 (B) outpatient 28.6 ± 13.3; inpatient 26.4 ± 16.3	NA	HM	No treatment	4 weeks/NR	1. NPI 2. MMSE 3. Barthel index 4. IADL
Monji et al. (2009)	15(10:5) →14(10:4)	(A) 80.8 ± 4.7 (B) 79.0 ± 2.0	(A) 10(2:8) (B) 5(0:5)	-AD (NINCDS-ADRDA, DSM-IV) -MMSE 6-23 (A) 15.1 ± 4.0 (B) 16.4 ± 3.5 -NPI≥6 on at least 1 of the delusions, hallucinations, agitation/aggression, disinhibition, irritability/lability or aberrant motor activity subscales after the treatment with Sulpiride 50 mg/day for 2 weeks (A) 26.7 ± 15.7 (B) 22.4 ± 12.8	NA	HM + (B)	Sulpiride 50 mg/day (modification according to patient's condition)	12 weeks/ NR	1. NPI 2. dose of sulpiride 3. MMSE 4. Barthel index

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TABLE 1 | (*Continued*) Basic characteristics of the randomized controlled trial.

Study ID	Sample size (included →analyzed)	Mean age (year)	Sex (M:F)	Population (Diagnosis)	Pattern identification	(A) Treatment intervention	(B) Control intervention	Treatment duration/ Follow-up	Outcome
Motohashi (2006)	52(28:24) →49(26:23)	(A) 80.00 ± 10.66 (B) 85.04 ± 9.64	(A) 28(9:19) (B) 24(7:17)	-VD (DSM-IV) -HDS-R≤20	NA	HM + (B)	Routine care (treating primary disease and symptomatic treatment)	8 weeks/NR	1. HDS-R 2. DAD 3. BEHAVE-AD 4. GBS 5. TER (cognitive symptom) 6. TCM symptom score 7. Serum lipoprotein(a) 8. Serum adrenalin 9. Serum noradrenalin 10. Serum dopamine
Okahara et al. (2010)	63(30:33) →61(29:32)	(A) 76.1 ± 8.1 (B) 77.1 ± 6.8	(A) 29(10:19) (B) 32(15:17)	-AD (NINCDS-ADRDA, DSM-IV, ICD-10) -MMSE (A) 18.3 ± 5.2 (B) 17.9 ± 5.5 -NPI (at least one symptom score of four or more in the NPI subscales) (A) 22.3 ± 10.4 (B) 21.9 ± 13.9 -SDS (A) 40.9 ± 7.7 (B) 43.7 ± 8.2 -HIS≤6	NA	HM + (B)	Donepezil (fixed dose during the study)	4 weeks/NR	1. NPI 2. MMSE 3. DAD 4. Zarit burden interview 5. SDS 6. Serum potassium
Pan et al. (2014)	98(49:49) →91(45:46)	(A) 57.2 ± 9.7 (B) 56.9 ± 10.2	(A) 45(28:17) (B) 46(27:19)	-AD (DSM-IV-TR) -MMSE 10-24 (A) 13.4 ± 1.8 (B) 14.1 ± 1.5	NA	HM	Placebo	20 weeks/ 5 weeks	1. MMSE 2. BEHAVE-AD 3. NPI 4. Actigraphy
Pu et al. (2014)	70(35:35) →70(35:35)	(A) 68.79 ± 7.99 (B) 71.34 ± 8.25	(A) 35(20:15) (B) 35(22:13)	-VD (CCMD-3) -MMSE≤26 (A) 11.36 ± 4.65 (B) 11.84 ± 4.52 -BEHAVE-AD≥8 (A) 17.80 ± 6.33 (B) 17.49 ± 6.58 -HIS>7	internal obstruction of static blood	HM	Oxcarbazepine 300–600 mg/day	8 weeks/NR	1. TER (BEHAVE-AD) 2. BEHAVE-AD 3. MMSE 4. ADL
Shen (2013)	110(55:55) →110(55:55)	(A) 75.3 (B) 74.6	(A) 55(32:23) (B) 55(34:21)	-AD or VD (CCMD-3) -MMSE (A) 15.4 ± 3.6 (B) 15.7 ± 4.1 -BEHAVE-AD≥8 (A) 18.4 ± 4.0 (B) 18.4 ± 3.69 -GDS<5	NA	HM	Olanzapine 2.5 mg/ day (modification up to 5–20 mg/day, according to patient's condition)	3 months/ NR	1. TER (BEHAVE-AD) 2. BEHAVE-AD 3. MMSE
Shen et al. (2018)	90(45:45)	(A) 64.36 ± 5.71	(A) 45(25:20)	-VD (2002 Criteria for Vascular Dementia of Neurology Branch of Chinese Medical Association)	NA	HM + (B)	Donepezil 5 mg/day	8 weeks/NR	1. TER (HAMD)

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TABLE 1 | (*Continued*) Basic characteristics of the randomized controlled trial.

Study ID	Sample size (included →analyzed)	Mean age (year)	Sex (M:F)	Population (Diagnosis)	Pattern identification	(A) Treatment intervention	(B) Control intervention	Treatment duration/ Follow-up	Outcome
	→90(45:45)	(B) 65.13 ± 6.14	(B) 45(26:19)	-Depression (CCMD-3) -MMSE (A) 10.56 ± 2.19 (B) 10.78 ± 2.24 -HAMD (A) 24.18 ± 2.67 (B) 24.35 ± 3.08					2. MMSE 3. ADL 4. HAMD 5. Serum dopamine 6. Serum BDNF 7. Serum Hcy
Shen et al. (2019)	100(50:50) →100(50:50)	(A) 69.2 ± 11.5 (B) 67.6 ± 10.6	(A) 50(33:17) (B) 50(35:15)	-VD (Criteria for Vascular Dementia of Neurology Branch of Chinese Medical Association) -Depression (CCMD-3) -MMSE (A) 17.69 ± 7.91 (B) 17.39 ± 6.28 -CSDD (A) 22.31 ± 4.22 (B) 21.86 ± 5.65 -CDR (mild to moderate dementia)	NA	HM + (B)	Oxiracetam 10 mg/ day (modification up to 20 mg/day, according to patient's response.)	8 weeks/NR	1. TER (HAMD) 2. CSDD 3. CDR 4. MMSE 5. ADL 6. Serum BDNF 7. Serum S100B 8. Serum norepinephrine 9. Serum dopamine 10. Serum 5-HT 11. Serum Hcy
Shi et al. (2020)	543(242: 241:60) →520(232: 233:55)	(A) 64.72 ± 9.18 (B1) 64.31 ± 9.99 (B2) 63.95 ± 9.15	(A) 232(154:78) (B1) 233(149:84) (B2) 55(35:20)	-VD (NINDS-AIREN) -MMSE 14-26 (A) 20.56 ± 3.36 (B1) 20.56 ± 3.24 (B2) 20.51 ± 2.97 -NPI (A) 5.31 ± 5.52 (B1) 5.35 ± 4.91 (B2) 5.40 ± 5.51 -HIS>7	NA	HM + Donepezil placebo	(B1) HM placebo + Donepezil (B2) HM placebo + Donepezil placebo	24 weeks/ NR	1. changes of VADAS-cog 2. improvement rate of CIBIC-plus 3. changes of NPI 4. changes of MMSE 5. changes of TMT-A 6. changes of TMT-B 7. changes of ADL 8. changes of CDT
Teranishi et al. (2013)	82(27:27:28) →76(26: 25:25)	(A) 83.50 ± 5.83 (B1) 80.72 ± 8.78 (B2) 83.20 ± 5.39	(A) 26(7:19) (B1) 25(9:16) (B2) 25(9:16)	-AD or VD or DLB (DSM-IV, NINCDS-ADRDA) -MMSE<19 (A) 4.42 ± 4.58 (B1) 5.16 ± 5.73 (B2) 4.48 ± 5.25 -NPI-NH (at least 1 symptom score of greater than 4 in NPI-NH) (A) 22.73 ± 14.30 (B1) 26.20 ± 15.77 (B2) 23.24 ± 15.53	NA	HM (Zopiclone (7.5–10 mg/day) and brotizolam (0.25 mg/day), if needed for insomnia)	(B1) Risperidone 0.5–2.0 mg/day (modification according to patient's condition) (B2) Fluvoxamine 25–200 mg/day (modification according to patient's condition) (Zopiclone (7.5–10 mg/day) and brotizolam (0.25 mg/ day), if needed for insomnia)	8 weeks/NR	1. NPI-NH 2. MMSE 3. FIM 4. DIEPSS

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TABLE 1 | (*Continued*) Basic characteristics of the randomized controlled trial.

Study ID	Sample size (included →analyzed)	Mean age (year)	Sex (M:F)	Population (Diagnosis)	Pattern identification	(A) Treatment intervention	(B) Control intervention	Treatment duration/ Follow-up	Outcome
Terasawa et al. (1997)	139(69:70) →119(55:64)	(A) 75.7 ± 8.9 (B) 77.6 ± 7.9	(A) 69(28:41) (B) 70(22:48)	-VD (DSM-III-R) -Carlo Loeb modified ischemic score≥5	NA	HM	Placebo	12 weeks/ NR	1. TER (overall symptom) 2. TER (subjective symptom) 3. TER (neurological symptom) 4. TER (psychiatric symptom) 5. TER (ADL) 6. TER (utility rating) 7. HDS-R
Yao et al. (2014)	80(40:40) →80(40:40)	(A) 71.3 ± 6.9 (B) 70.1 ± 8.1	(A) 40(23:17) (B) 40(21:19)	-VD (Textbook in neurology) -CDR (A) 1.5 ± 0.5 (B) 1.5 ± 0.5 -HAMD (A) 1.3 ± 0.6 (B) 1.4 ± 0.6	internal obstruction of static blood	HM + (B)	Clopidogrel 75 mg/day	3 months/ NR	1. ADL 2. CDR 3. HAMD 4. TER (ADL, CDR, HAMD)
Zhang (2012)	80(40:40) →80(40:40)	(A) 74.55 ± 6.30 (B) 74.43 ± 6.45	NR	-AD or VD (CCMD-3) -MMSE<24 -BEHAVE-AD≥8 (A) 16.2 ± 7.8 (B) 16.1 ± 7.6	NA	HM	Haloperidol 1 mg/day (modification up to 4–10 mg/day within 2 weeks)	8 weeks/NR	1. TER (BEHAVE-AD) 2. BEHAVE-AD
Zhang et al. (2015b)	144(72:72) →144(72:72)	(A) 72.79 ± 6.76 (B) 72.97 ± 6.59	(A) 72(26:46) (B) 72(29:43)	-AD (DSM-IV) -MMSE (A) 20.49 ± 4.29 (B) 19.82 ± 3.54 -NPI (A) 1.50 ± 2.96 (B) 1.35 ± 2.04 -HIS≤4 -HAMD≤7 -CDR 1	NA	HM + Donepezil placebo	HM placebo + Donepezil	24 weeks/ 24 weeks	1. ADAS-cog 2. MMSE 3. ADL 4. NPI
Zhang et al. (2018)	94(47:47 →94(47:47)	(A) 67.2 ± 6.9 (B) 68.1 ± 6.9	(A) 47(23:24) (B) 47(22:25)	-AD (NINCDS-ADRDA) -CSDD>8 -HAMA>14 -HAMD>17	Liver depression and spleen deficiency	HM + (B)	Buspirone 15 mg/day (modification up to 30 mg/day), Sertraline 100 mg/day	6 weeks/NR	1. CSDD 2. HAMA 3. HAMD 4. GQOLI-74 5. TESS
Zhou (2015a)	40(20:20) →36(18:18)	(A) 73.89 ± 4.31 (B) 73.61 ± 3.73	(A) 18(7:11) (B) 18(6:12)	-AD (NINCDS-ADRDA) -Depression (NIMH-dAD, DSM-IV-TR) -CSDD>8 (A) 15.44 ± 2.52 (B) 15.11 ± 2.93 -HIS≤4 -MMSE 10~24 -CDR 1 or 2	Liver depression and spleen deficiency	HM + Huperzine A 200ug/day	Escitalopram 5 mg/ day (modification up to 10 mg/day) + Huperzine A 200ug/day	6 weeks/NR	1. CSDD 2. TER (CSDD) 3. SF-36 4. TCM symptom score 5. TER (TCM symptom score)

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TABLE 1 | (*Continued*) Basic characteristics of the randomized controlled trial.

Study ID	Sample size (included →analyzed)	Mean age (year)	Sex (M:F)	Population (Diagnosis)	Pattern identification	(A) Treatment intervention	(B) Control intervention	Treatment duration/ Follow-up	Outcome
Zhou (2015b)	80(40:40) →80(40:40)	(A) 54.5 ± 6.1 (B) 55.5 ± 6.7	(A) 40(21:19) (B) 40(19:21)	-AD (DSM-IV) -MMSE (A) 16.07 ± 2.44 (B) 16.77 ± 3.16 -Depression (CCMD-3)	NA	HM + Donepezil 5 mg/day	Escitalopram 10 mg/day + Donepezil 5 mg/day	3 months/ NR	1. HAMD 2. MMSE
Zhou (2018)	80(40:40) →80(40:40)	72.35 ± 3.24	80(44:36)	-AD (CCMD) -MMSE (A) 22.93 ± 2.41 (B) 22.86 ± 2.27	NA	HM	Donepezil 10 mg/day, Magnesium valproate sustained-release 500 mg/day	1 month/NR	1. TER (MMSE) 2. MMSE 3. PSQI
Zhou et al. (2019)	60(30:30) →60(30:30)	(A) 62.20 ± 5.56 (B) 63.32 ± 5.18	(A) 30(17:13) (B) 30(14:16)	-VD (NINDS-AIREN) -MMSE≤23 (A) 16.83 ± 2.10 (B) 17.23 ± 2.43 -NPI (A) 44.00 ± 13.83 (B) 46.47 ± 13.61 -HIS≥7 -CDR (mild to moderate dementia)	qi deficiency, phlegm, and stasis	HM + (B)	Donepezil 5 mg/day	8 weeks/NR	1. TER (TCM symptom score) 2. MMSE 3. ADL 4. NPI 5. TCM symptom score 6. Plasma hs-CRP 7. Plasma TNF-α 8. Plasma IL-6 9. Plasma Hcy 10. Plasma MDA 11. Plasma SOD
Zuo (2017)	56(30:26) →56(30:26)	(A) 66.5 ± 12.3 (B) 67.6 ± 10.8	(A) 30(25:5) (B) 26(20:6)	-AD (IWG-2 criteria) -Depression (HAMD≥17)	NA	HM	Sertraline 50 mg/day (modification up to 100 mg/day)	60 days/NR	1. TER (HAMD) 2. HAMD

ACE-R, Addenbrooke cognitive examination revised; AD, Alzheimer's disease; ADAS-cog, Alzheimer's disease assessment scale–cognitive subscale; ADL, activities of daily living; BDNF, brain-derived neurotrophic factor; BEHAVE-AD, behavioral pathology in Alzheimer's disease rating scale; BPRS, brief psychiatric rating scale; CCMD, Chinese classification of mental disorders; CDR, clinical dementia rating; CDT, clock drawing test; CIBIC-plus, clinician's interview-based impression of change-plus caregiver information; CRP, C-reactive protein; CSDD, Cornell scale for depression in dementia; DAD, disability assessment of dementia; DIEPSS, drug-induced extra-pyramidal symptoms scale; DLB, dementia with Lewy bodies; DSM, diagnostic and statistical manual of mental disorders; FIM, functional independence measure; GBS, Gottfries-Bråne-Steen; GDS, global deterioration scale; GQOLI-74, generic quality of life inventory-74; HAMA, Hamilton anxiety rating scale; HAMD, Hamilton depression rating scale; Hcy, homocysteine; HDS, Hasegawa's dementia scale; HDS-R, the revised Hasegawa's dementia scale; HIS, Hachinski ischemia score; HM, herbal medicine; IADL, instrumental activities of daily living; ICD, the international statistical classification of diseases and related health problems; IL, interleukin; IWG, international working group; MDA, malondialdehyde; MMSE, mini-mental state examination; MoCA, Montreal cognitive assessment; NA, not applicable; NIMH-dAD, National Institute of Mental Health criteria for depression in Alzheimer's disease; NINCDS-ADRDA, national institute of neurological and communicative diseases and stroke/Alzheimer's disease and related disorders association; NINDS-AIREN, national Institute of neurological disorders and stroke and association internationale pour la Recherché et l'Enseignement en neurosciences; NPI, neuropsychiatric inventory; NPI-NH, neuropsychiatric inventory-nursing home; NPI-Q, neuropsychiatric inventory-questionnaire; NR, not recorded; PSQI, Pittsburgh sleep quality index; SD, senile dementia; SDS, self-rating depression scale; SDSD, dementia syndrome type scale; SF-36, 36-item short form survey; SOD, superoxide dismutase; TCM, traditional Chinese medicine; TER, total effective rate; TESS, treatment emergent symptom scale; TMT, trail making test; TNF, tumor necrosis factor; VADAS-cog, vascular dementia assessment scale-cognitive subscale; VD, vascular dementia

et al., 2013; Teranishi et al., 2013; Furukawa et al., 2017), followed by Xiaoyaosan (four studies) (Zhou, 2015; Zhou and Wei, 2015; Shen et al., 2018; Shen et al., 2019) and Liuweidihuang pill (three studies (Shen, 2013; Du et al., 2015; Gu et al., 2018). In terms of dosage form, powder was most often used in 18 studies (Terasawa et al., 1997; Iwasaki et al., 2005; Shinno et al., 2007; Mizukami et al., 2009; Monji et al., 2009; Hayashi et al., 2010; Kawanabe et al., 2010; Okahara et al., 2010; Iwasaki et al., 2012; Nagata et al., 2012; Sumiyoshi et al., 2013; Teranishi et al., 2013; Zhou and Wei, 2015; Kudoh et al., 2016; Furukawa et al., 2017; Ohsawa et al., 2017; Meguro and Yamaguchi, 2018; Manabe, 2020), followed by decoction (16 studies) (Guo et al., 2011a; Guo et al., 2011b; Yang et al., 2012; Zhang, 2012; Pu et al., 2014; Yao et al., 2014; Zhang et al., 2015a; Hu et al., 2015; Zhou, 2015; Zuo, 2017; Han, 2018; Li et al., 2018; Zhou, 2018; Huang and Xu, 2019; Zhu et al., 2019; Li, 2020), granules (seven studies) (Motohashi, 2006; Xu et al., 2007; Chen et al., 2013; Liu et al., 2015; Xu, 2018; Chen, 2020; Shi et al., 2020), pill (five studies) (Shen, 2013; Du et al., 2015; Gu et al., 2018; Shen et al., 2018; Shen et al., 2019), and capsules (four studies) (Chen et al., 1997; Lin et al., 2016; Fang et al., 2018; Zhang et al., 2018) (**Supplementary Material S4**).

RoB in Studies

For RCTs, a total of 19 (Du et al., 2015; Furukawa et al., 2017; Guo et al., 2011a; Han, 2018; Hu et al., 2015; Li, 2020; Li et al., 2018; Lin et al., 2016; Mizukami et al., 2009; Monji et al., 2009; Pan et al., 2014; Pu et al., 2014; Shen, 2013; Shen et al., 2018; Shi et al., 2020; Teranishi et al., 2013; Zhang et al., 2015a; Zhang et al., 2018; Zhou, 2015) and two studies (Shi et al., 2020; Zhang et al., 2015a) were evaluated as having a low RoB in the corresponding domain, mentioning the appropriate random sequence generation method and allocation concealment, respectively. Each of four (Pan et al., 2014; Shi et al., 2020; Teranishi et al., 2013; Zhang et al., 2015a) and five studies (Fang et al., 2018; Furukawa et al., 2017; Shi et al., 2020; Teranishi et al., 2013; Zhang et al., 2015a) were evaluated with low risk of performance or detection bias by appropriately performing blinding of participants, personnel, or outcome assessors. In one study (Terasawa et al., 1997), the number of dropouts in each group was not described in detail, and the lack of outcomes related to BPSD was evaluated as a high risk of attrition and reporting bias. Two studies (Chen et al., 1997; Motohashi, 2006) were evaluated as having an unclear risk of other bias because there was no information on the homogeneity of baseline clinical characteristics between the two groups (**Figure 2**). In one CCT (Xu, 2018), the treatment or control group or outcome measures were not properly specified. In 12 before–after studies (Iwasaki et al., 2005; Xu et al., 2007; Shinno et al., 2008; Hayashi et al., 2010; Kawanabe et al., 2010; Guo et al., 2011b; Iwasaki et al., 2012; Nagata et al., 2012; Yang et al., 2012; Sumiyoshi et al., 2013; Ohsawa et al., 2017; Manabe, 2020), study questions, eligibility criteria for the study population, interventions, and outcome measures were clearly stated in most studies. However, blinding of outcome assessors was not reported in all studies, and only two studies (Iwasaki et al., 2005; Manabe, 2020) provided individual-level data (**Supplementary Material S5**).

Effectiveness (or Efficacy) and Safety of HM in Included RCTs

HM as a Monotherapy

Compared to psychotropic drugs, the severity of BPSD symptoms evaluated by BEHAVE-AD was not significantly different between the two groups (MD = −0.36; 95% CI: −1.09 to 0.36), regardless of the treatment duration. However, as a result of subgroup analysis according to the severity and type of dementia, in the case of severe dementia and vascular dementia, BEHAVE-AD was significantly reduced in the HM group (MD = −2.34, 95% CI: −4.15 to −0.53). The Hamilton depression rating scale (HAMD) was significantly improved in the HM group (MD = −2.86; 95% CI: −3.85 to −1.87), but there was no significant difference between the two groups in TER calculated based on BPSD symptoms (RR = 1.05, 95% CI: 0.95–1.16). To evaluate the severity of BPSD, NPI (Zhang et al., 2015a), NPI-nursing home (Teranishi et al., 2013), and the Cornell scale for depression in dementia (CSDD) (Zhou, 2015) were evaluated in one each, and there was no significant difference between the two groups in all studies. In addition, AE occurred significantly less in the HM group (RR = 0.40, 95% CI: 0.25–0.64), regardless of the treatment duration (**Table 2**). However, when the sensitivity analysis was performed on studies with low risk of performance and detection bias, there was no significant difference in the meta-analysis of AE between the two groups (RR = 0.83; 95% CI: 0.27–2.61). Overall, severe AEs related to HM were rarely reported, but hypokalemia in HMs containing licorice, such as Yokukansan, required attention (**Supplementary Material S6**).

When comparing HM and placebo, in one study (Furukawa et al., 2017), there was no significant difference between the two groups in the NPI-questionnaire. However, in another study (Pan et al., 2014) that measured the severity of BPSD symptoms, the HM group showed significant improvement in hallucinations, activity disturbances, aggressiveness, and anxieties and phobias of BEHAVE-AD, and in delusions, hallucinations, agitation, aberrant motor behavior, and sleep disturbance of NPI ($p < 0.05$), although there were no significant differences between the two groups in other domains. In a study comparing the group administered HM and donepezil placebo with the group administered with HM placebo and donepezil placebo (Shi et al., 2020), it was reported that the change in the NPI total score after treatment was significantly greater in the group administered with HM and donepezil placebo ($p < 0.05$). Finally, a study that evaluated TER based on psychiatric symptoms reported that TER of the HM group was significantly higher than that of the placebo group (Terasawa et al., 1997). There was no significant difference in the incidence of AE between the two groups (RR = 2.67; 95% CI: 0.93–7.65) (**Table 2**). Even when the sensitivity analysis was performed only with studies with a low risk of performance and detection bias, this result was not affected.

When comparing HM and anti-dementia drugs, one study (Zhou, 2018) showed that the Pittsburgh Sleep Quality Index (PSQI) was significantly reduced in the HM group ($p < 0.05$). In one study comparing HM and no treatment (Mizukami et al., 2009), the severity of BPSD symptoms measured by NPI after 4 weeks of treatment was significantly improved in the HM group

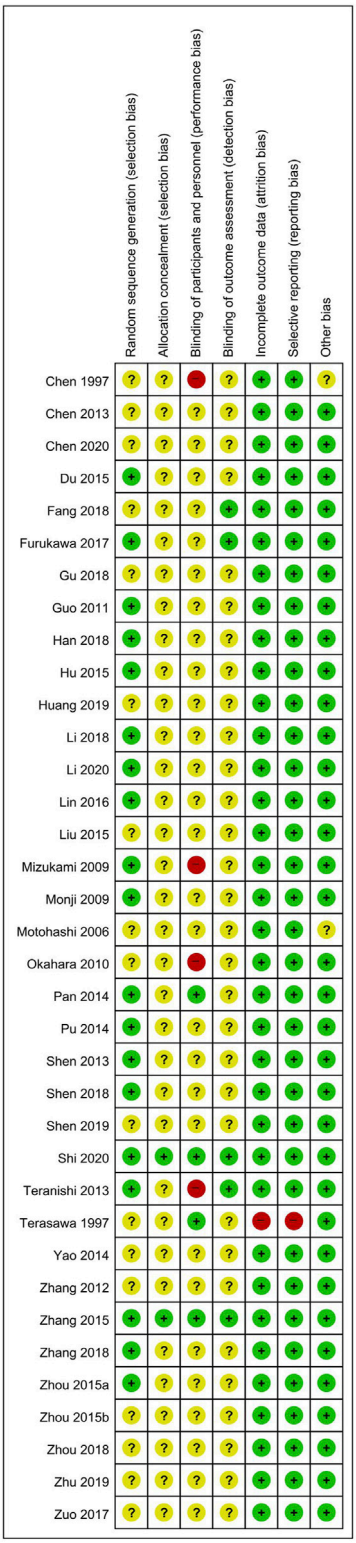

FIGURE 2 | Risk of bias for all included studies. Low, unclear, and high risk, respectively, are represented with the following symbols: "+", "?", and "-".

($p < 0.05$), but the Barthel index or IADL was not significantly different between the two groups. In one study comparing HM and hydergine (Chen et al., 1997), there was no significant difference in the HAMD score after treatment.

HM as an Adjunctive Therapy

When HM was additionally used for psychotropic drugs, the BEHAVE-AD (MD = −3.48, 95% CI: −3.96 to −2.99) and TER calculated based on BPSD symptoms (RR = 1.16, 95% CI: 1.05–1.29) significantly improved, compared with psychotropic drugs alone. In addition, the incidence of AE was also significantly lower in the HM group (RR = 0.71; 95% CI: 0.50–0.99; **Table 2**). When HM was additionally used, one study Gu et al. (2018) reported that the BPRS score improved significantly ($p < 0.05$), and another study Zhang et al. (2018) reported that QoL and the severity of BPSD evaluated by CSDD, Hamilton Anxiety Rating Scale, and HAMD significantly improved, and the frequency of side effects evaluated by treatment emergent symptom scale was significantly reduced ($p < 0.05$, all).

When HM was additionally used as an anti-dementia drug, the severity of BPSD symptoms measured by BEHAVE-AD (MD = −2.81, 95% CI: −3.17 to −2.45), NPI (MD = −3.23, 95% CI: −4.06 to −2.40), and HAMD (MD = −4.92, 95% CI: −5.48 to −4.37) significantly improved, compared with anti-dementia drugs alone. The TER calculated based on BPSD symptoms (RR = 1.29, 95% CI: 1.13–1.47) and Barthel index (MD = 3.42, 95% CI: 2.67–4.16) also significantly improved in the HM group. The severity of BPSD symptoms was evaluated using PSQI(30) ($p < 0.05$) and NPI-questionnaire (Hu et al., 2015) ($p < 0.01$) in each study, and both showed significantly improved results in the HM group. In one study (Okahara et al., 2010), the degree of depression and burden of caregivers after treatment were reported through a self-rating depression scale and a Zarit burden interview, respectively, but there was no significant difference between the two groups. The frequency of AE was also significantly lower in the HM group (RR = 0.50, 95% CI: 0.28–0.88), although there was no consistent result according to the subgroups (**Table 2**,**Supplementary Material S6**).

Furthermore, when HM was additionally used, there was no significant difference in the CSDD score compared with the oxiracetam alone (Shen et al., 2019). However, HAMD significantly improved ($p < 0.05$) when HM was additionally used to clopidogrel in one study (Yao et al., 2014).

Publication Bias

Since there was no meta-analysis that included more than ten studies, we could not assess the publication bias using a funnel plot.

Results From Other Included Studies

In addition to the included RCTs, all other studies (Iwasaki et al., 2005; Shinno et al., 2007; Xu et al., 2007; Shinno et al., 2008; Hayashi et al., 2010; Kawanabe et al., 2010; Guo et al., 2011b; Iwasaki et al., 2012; Nagata et al., 2012; Yang et al., 2012; Sumiyoshi et al., 2013; Kudoh et al., 2016; Ohsawa et al., 2017; Meguro and Yamaguchi, 2018; Xu, 2018; Manabe, 2020) have reported that HM improved BPSD in at least one indicator.

TABLE 2 | Effect estimates of meta-analysis.

Outcomes	Subgroup	No. RCTs	No. participants	Effect estimate MD/RR (95%CI)	I^2 value (%)	Model
Herbal medicine vs. psychotropic drugs						
BEHAVE-AD	Total	4	320	MD -0.36 [-1.09, 0.36]	45	Fixed
Dementia severity/type	Moderate/2 or more dementia	3	250	MD 0.01 [-0.78, 0.80]	0	Fixed
	Severe/VD	1	70	MD -2.34 [-4.15, -0.53]	NA	Fixed
Treatment duration	≤1 month	1	60	MD 0.20 [-3.17, 3.57]	NA	Fixed
	1 < month ≤ 2	2	150	MD -0.63 [-1.57, 0.31]	79	Fixed
	2 < month ≤ 6	1	110	MD 0.00 [-1.20, 1.20]	NA	Fixed
HAMD	Total (AD)	2	136	MD -2.86 [-3.85, -1.87]	87	Fixed
Dementia severity/treatment duration	Unclear/1 < month ≤ 2	1	56	MD -4.28 [-5.70, -2.86]	NA	Fixed
	Moderate/2 < month ≤ 6	1	80	MD -1.50 [-2.89, -0.11]	NA	Fixed
TER (BPSD symptom)	Total	5	332	RR 1.05 [0.95, 1.16]	45	Fixed
Dementia severity/type	Moderate/2 or more dementia	2	170	RR 0.95 [0.85, 1.06]	0	Fixed
	Severe/VD	1	70	RR 1.15 [0.93, 1.43]	NA	Fixed
	Unclear/AD	2	92	RR 1.22 [0.92, 1.61]	66	Fixed
Treatment duration	≤1 month	1	60	RR 0.96 [0.83, 1.12]	NA	Fixed
	1 < month ≤ 2	3	162	RR 1.18 [0.99, 1.41]	31	Fixed
	2 < month≤6	1	110	RR 0.94 [0.81, 1.09]	NA	Fixed
Adverse event	Total	4	360	RR 0.40 [0.25, 0.64]	0	Fixed
Dementia severity	Mild	1	144	RR 0.83 [0.27, 2.61]	NA	Fixed
	Moderate	1	110	RR 0.35 [0.15, 0.83]	NA	Fixed
	Severe	1	70	RR 0.35 [0.18, 0.67]	NA	Fixed
	Unclear	1	36	RR 0.20 [0.01, 3.89]	NA	Fixed
Dementia type	AD	2	180	RR 0.65 [0.23, 1.82]	0	Fixed
	VD	1	70	RR 0.35 [0.18, 0.67]	NA	Fixed
	2 or more	1	110	RR 0.35 [0.15, 0.83]	NA	Fixed
Treatment duration	1 < month ≤ 2	2	106	RR 0.33 [0.18, 0.63]	0	Fixed
	2 < month ≤ 6	2	254	RR 0.48 [0.25, 0.93]	28	Fixed
Herbal medicine vs. placebo						
Adverse event	Total	4	662	RR 2.67 [0.93, 7.65]	0	Fixed
Dementia severity	Mild	1	287	RR 0.95 [0.11, 8.32]	NA	Fixed
	Moderate	2	236	RR 5.60 [0.69, 45.36]	NA	Fixed
	Unclear	1	139	RR 2.54 [0.51, 12.63]	NA	Fixed
Dementia type	AD	2	236	RR 5.60 [0.69, 45.36]	NA	Fixed
	VD	2	426	RR 1.82 [0.52, 6.42]	0	Fixed
Treatment duration	≤1 month	1	145	RR 5.60 [0.69, 45.36]	NA	Fixed
	2 < month ≤ 6	3	517	RR 1.82 [0.52, 6.42]	0	Fixed
Herbal medicine + psychotropic drugs vs. psychotropic drugs						
BEHAVE-AD	Total	3	274	MD -3.48 [-3.96, -2.99]	87	Fixed
Dementia severity/treatment duration	Moderate/1 < month ≤ 2	2	174	MD -3.48 [-4.07, -2.89]	93	Fixed
	Unclear/2 < month ≤ 6	1	100	MD -3.47 [-4.30, -2.64]	NA	Fixed
Dementia type	AD	2	184	MD -2.96 [-3.52, -2.39]	63	Fixed
	SD	1	90	MD -4.90 [-5.83, -3.97]	NA	Fixed
TER (BPSD symptom)	Total	4	354	RR 1.16 [1.05, 1.29]	0	Fixed
Dementia severity/treatment duration	Moderate/1 < month ≤ 2	2	174	RR 1.19 [1.04, 1.37]	0	Fixed
	Unclear/2 < month ≤ 6	2	180	RR 1.13 [0.98, 1.31]	0	Fixed
Dementia type	AD	3	264	RR 1.16 [1.03, 1.30]	0	Fixed
	SD	1	90	RR 1.18 [0.96, 1.46]	NA	Fixed
Adverse event	Total	5	369	RR 0.71 [0.50, 0.99]	49	Fixed
Dementia severity	Moderate	3	189	RR 0.79 [0.48, 1.31]	49	Fixed
	Unclear	2	180	RR 0.64 [0.40, 1.01]	75	Fixed
Dementia type	AD	4	279	RR 0.62 [0.42, 0.92]	52	Fixed
	SD	1	90	RR 1.09 [0.54, 2.21]	NA	Fixed
Treatment duration	1 < month ≤ 2	2	174	RR 0.72 [0.43, 1.20]	62	Fixed
	2 < month ≤ 6	3	195	RR 0.70 [0.45, 1.09]	62	Fixed
Herbal medicine + anti-dementia drugs vs. anti-dementia drugs						
BEHAVE-AD	Total	4	333	MD -2.81 [-3.17, -2.45]	89	Fixed
Dementia severity	Moderate	2	168	MD -3.06 [-3.44, -2.68]	85	Fixed

(Continued on following page)

TABLE 2 | (*Continued*) Effect estimates of meta-analysis.

Outcomes	Subgroup	No. RCTs	No. participants	Effect estimate MD/RR (95%CI)	I^2 value (%)	Model
	Unclear	2	165	MD -0.96 [-2.00, 0.08]	85	Fixed
Dementia type	AD	3	228	MD -3.04 [-3.42, -2.67]	70	Fixed
	2 or more	1	105	MD 0.10 [-1.22, 1.42]	NA	Fixed
Treatment duration	≤1 month	2	142	MD -3.20 [-3.60, -2.81]	0	Fixed
	2 < month ≤ 6	2	191	MD -0.84 [-1.72, 0.04]	72	Fixed
NPI	Total (Moderate)	4	264	MD -3.23 [-4.06, -2.40]	97	Fixed
Dementia type	VD	1	60	MD -5.76 [-10.67, -0.85]	NA	Fixed
	AD	3	204	MD -3.15 [-4.00, -2.31]	98	Fixed
Treatment duration	≤1 month	1	61	MD -5.40 [-12.48, 1.68]	NA	Fixed
	1 < month ≤ 2	1	60	MD -5.76 [-10.67, -0.85]	NA	Fixed
	2 < month ≤ 6	2	143	MD -3.12 [-3.97, -2.27]	99	Fixed
HAMD	Total (1 < month ≤ 2)	2	180	MD -4.92 [-5.48, -4.37]	83	Fixed
Dementia severity/type	Severe/VD	1	90	MD -5.30 [-5.94, -4.66]	NA	Fixed
	Unclear/AD	1	90	MD -3.70 [-4.85, -2.55]	NA	Fixed
TER (BPSD symptom)	Total	3	255	RR 1.29 [1.13, 1.47]	65	Fixed
Dementia severity	Severe	1	90	RR 1.28 [1.04, 1.58]	NA	Fixed
	Unclear	2	165	RR 1.29 [1.09, 1.54]	83	Fixed
Dementia type/treatment duration	AD/≤1 month	1	60	RR 1.80 [1.23, 2.62]	NA	Fixed
	VD/1 < month ≤ 2	1	90	RR 1.28 [1.04, 1.58]	NA	Fixed
	2 or more/2 < month ≤ 6	1	105	RR 1.11 [0.93, 1.33]	NA	Fixed
Barthel index	Total (2 < month ≤ 6)	2	162	MD 3.42 [2.67, 4.16]	99	Fixed
Dementia severity/type	Moderate/AD	1	57	MD 1.20 [0.34, 2.06]	NA	Fixed
	Unclear/2 or more	1	105	MD 10.44 [8.91, 11.97]	NA	Fixed
Adverse event	Total	5	376	RR 0.50 [0.28, 0.88]	15	Fixed
Dementia severity	Moderate	2	121	RR 2.00 [0.19, 20.90]	NA	Fixed
	Severe	1	90	RR 0.31 [0.11, 0.87]	NA	Fixed
	Unclear	2	165	RR 0.56 [0.27, 1.16]	26	Fixed
Dementia type/treatment duration	AD/≤1 month	2	121	RR 2.00 [0.19, 20.90]	NA	Fixed
	VD/1 < month ≤ 2	2	150	RR 0.43 [0.17, 1.05]	51	Fixed
	2 or more/2 < month ≤ 6	1	105	RR 0.46 [0.21, 1.03]	NA	Fixed

AD, Alzheimer's disease; BEHAVE-AD, behavioral pathology in Alzheimer's disease rating scale; BPSD, behavioral and psychological symptoms of dementia; CI, confidence interval; HAMD, Hamilton depression rating scale; MD, mean difference; NA, not applicable; NPI, neuropsychiatric inventory; RCT, randomized controlled trial; RR, risk ratio; SD, senile dementia; TER, total effective rate; VD, vascular dementia.

However, considering the design of the study, these studies were not included as evidence for analyzing the effectiveness or efficacy of HM for BPSD but reviewed in terms of the current status of research in this field. More information can be found in **Supplementary Material S3, S6**.

DISCUSSION

Summary of Evidence

In this systematic review, the most comprehensive review and meta-analysis to date was conducted to analyze the effectiveness (or efficacy), safety, and research status of HM for BPSD. According to the meta-analysis, HM did not show statistically significant differences from psychotropic drugs in the effectiveness of BPSD evaluated as BEHAVE-AD or TER or in the subgroup analysis of dementia severity, dementia type, or treatment duration. However, a few studies reported that HM showed statistically significant improvement in patients with Alzheimer's disease compared to psychotropic drugs in HAMD. In addition, HM appeared to be safer compared to psychotropic drugs in terms of the incidence of AEs. Comparisons between HM and placebo in four studies did not have homogeneous outcomes in the meta-analysis. Of the four studies, three showed significant differences between the HM and placebo groups in improving BPSD symptoms but not in the one remaining study. The incidence of AE was not significantly different between the groups, and these results did not change according to the subgroup analysis on dementia severity, dementia type, or treatment duration. When HM was compared to anti-dementia drugs or no treatment, there were statistically significant benefits in improving sleep quality assessed by PSQI or BPSD symptoms assessed by NPI in a study, respectively. When HM was used as an adjunctive therapy, it showed the most consistent benefit. When HM was used in combination with psychotropic or anti-dementia drugs, there were statistically significant benefits in BEHAVE-AD, NPI, HAMD, TER based on BPSD symptoms, incidence of AEs, and Barthel index, compared to monotherapy with psychotropic or anti-dementia drugs. The methodological quality of the RCTs included in this systematic review was not optimal overall. In particular, allocation concealment and blinding domains were evaluated as unclear in most studies.

Clinical Implications

HM is an EATM modality that has long been used in Asian countries for health improvement and disease treatment. The results of this systematic review and meta-analysis provided limited evidence that HM may be associated with additional benefits in BPSD treatment, particularly when used as an adjunct to conventional medications, including psychotropic and anti-dementia drugs. Although the clinical evidence supporting the effectiveness (or efficacy) and safety of HM for BSPD is insufficient, this topic has clinical relevance considering that many elderly patients already use prescription drugs and HM in combination (de Souza Silva et al., 2014; Agbabiaka et al., 2017). Additionally, the use of HMs is not limited to EATM. Herbs used in other traditions, such as *Ginkgo biloba*, *Withania somnifera*, *Panax ginseng*, and *Curcuma longa,* and some phytochemicals have also shown promising results in the treatment of dementia (Alzobaidi et al., 2021). Currently, studies are focused on drug delivery, such as improving the potential anti-dementia effect of HM by using a targeted nanocarrier system (Moradi et al., 2020; Singh et al., 2021). Any current or future studies that explore the therapeutic potential of HM for dementia should be encouraged, as these can provide valuable insight in the field.

Although not within the scope of this review, HM is also used to delay cognitive decline, a core symptom of dementia, and its mechanisms are being studied to be related to mechanisms such as anti-inflammatory, antioxidative, and antiapoptotic activity (Tewari et al., 2018). However, the underlying mechanism of HM for the core and associated symptoms of dementia is yet unclear, and it may be related to some challenges including non-uniform chemical composition, non-standardized ratio of herb ingredients, and its multi-component and multi-target mechanism (Zhou et al., 2019). Moreover, there are safety issues associated with HM, such as lack of safety monitoring and potential interactions with conventional pharmaceuticals (Ekor, 2014). Fortunately, for some standardized HMs, such as Yokukansan, underlying therapeutic mechanisms for dementia (Takeyoshi et al., 2016), potential interactions with conventional medications (Soraoka et al., 2016) and safety issues have been documented (Shimada et al., 2017). Similarly, a database of some potential herb-drug interactions relevant to the management of cognitive impairment has been recently developed. It provides the pharmacological interactions of 170 bio-actives with 10 commonly-used drugs (Auxtero et al., 2021). However, other heterogeneous HMs are also used in clinical practice and their safety profiles need further clarification. Spontaneous reporting systems and active pharmacovigilance for the use of HMs should be encouraged with stringent oversight by a national-level regulatory body to ensure patient safety and satisfaction (Zhang et al., 2015b).

In summary, in order for HM to be seamlessly integrated into the conventional medical system in the management of dementia, particularly to treat BPSD, the use of standardized HM with well-managed quality should be encouraged, and the underlying mechanisms and possible interactions with conventional pharmaceuticals should be further investigated. It should also be used by health care professionals in clinics or hospital-based settings for meticulous effectiveness and safety monitoring.

Strengths and Limitations

This systematic review comprehensively reviewed the studies published to date on this issue and summarized the clinical evidence supporting the effectiveness and safety of HM in the management of BPSD. Considering the limitations of psychotropic drugs in the management of BPSD, particularly in the elderly, and many elderly patients already use HM, this topic has great clinical relevance. Our study highlights the limited evidence of HM for BPSD management and discusses the future directions necessary for HM to be integrated into conventional dementia care systems as an adjuvant therapy.

The findings of this systematic review should be interpreted with careful consideration of some limitations (Wimo et al., 2017). Although this review collected clinical evidence of HM for BPSD as the most comprehensive, the number of studies included in each meta-analysis was less than six because the studies included were heterogeneous. In particular, some standardized HMs, such as Yokukansan, existed, but most studies used HMs of heterogeneous composition. Although EATM is a medicine system that emphasizes holistic and individualized approaches (Fung and Linn, 2015), the use of standardized HM is emphasized in order to establish an effective HM use strategy for BPSD treatment and to confirm its expected effectiveness and safety. In addition, in order to accumulate robust clinical evidence of HM for BPSD management, the design of dementia severity, dementia type, BPSD severity, and treatment duration of subjects should be homogeneous (Ohno et al., 2019). In the protocol of this review (Kwon et al., 2021), the subgroup analysis was planned according to the severity of baseline BPSD of participants, but this subgroup analysis was not possible because of the heterogeneity of the indicators. However, since psychotropic drugs, such as antipsychotics, are generally more recommended for severe BPSD compared to safe non-pharmacological therapy (Masopust et al., 2018), finding other safe alternatives, including HM, in patients with severe BPSD, is necessary (Cerejeira et al., 2012). Since only a few studies were included in each meta-analysis, evaluation of publication bias through funnel plots was not possible. However, most studies included in the analysis were conducted and reported in China, which suggests a potential publication bias in the results. Although HM is mainly used in Asian countries as an EATM modality, rigorous clinical trials conducted in Taiwan and Korea, in addition to China and Japan, are encouraged to address this issue (van der Linde et al., 2016). None of the included studies reported the results of economic value related to HM for BPSD. Dementia causes a huge socioeconomic burden worldwide, and BPSD is a major contributing factor (Cerejeira et al., 2012). Therefore, effective alternatives to BPSD in the future require cost-effectiveness, effectiveness, and safety. Considering that the cost-effectiveness of HM is being studied for other clinical topics, such as chronic low back pain (Sung et al., 2019), further clinical research on HM for BPSD should encompass economic evaluation.

CONCLUSION

According to the findings of this review, HM may be associated with additional benefits in BPSD treatment, particularly when used as an adjunct to conventional medications, including psychotropic and anti-dementia drugs. However, considering the methodological quality of the included RCTs, this clinical evidence is not robust. In addition, the heterogeneity of HMs used in each study encourages the use of standardized HMs in the future. Nevertheless, dementia is a global health concern, and considering the limitations of conventional psychotropic drugs for BPSD, a major cause of the disease burden, HM appears to be a promising complementary therapy that warrants further research.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

The Conceptualization: C-YK. Funding acquisition: C-YK. Methodology: C-YK and BL. Supervision: C-YK. Writing–original draft: C-YK and BL.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.713287/full#supplementary-material

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Active Fraction Combination From Liuwei Dihuang Decoction Improves Adult Hippocampal Neurogenesis and Neurogenic Microenvironment in Cranially Irradiated Mice

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Background: Cranial radiotherapy is clinically used in the treatment of brain tumours; however, the consequent cognitive and emotional dysfunctions seriously impair the life quality of patients. LW-AFC, an active fraction combination extracted from classical traditional Chinese medicine prescription Liuwei Dihuang decoction, can improve cognitive and emotional dysfunctions in many animal models; however, the protective effect of LW-AFC on cranial irradiation–induced cognitive and emotional dysfunctions has not been reported. Recent studies indicate that impairment of adult hippocampal neurogenesis (AHN) and alterations of the neurogenic microenvironment in the hippocampus constitute critical factors in cognitive and emotional dysfunctions following cranial irradiation. Here, our research further investigated the potential protective effects and mechanisms of LW-AFC on cranial irradiation–induced cognitive and emotional dysfunctions in mice.

Methods: LW-AFC (1.6 g/kg) was intragastrically administered to mice for 14 days before cranial irradiation (7 Gy γ-ray). AHN was examined by quantifying the number of proliferative neural stem cells and immature neurons in the dorsal and ventral hippocampus. The contextual fear conditioning test, open field test, and tail suspension test were used to assess cognitive and emotional functions in mice. To detect the change of the neurogenic microenvironment, colorimetry and multiplex bead analysis were performed to measure the level of oxidative stress, neurotrophic and growth factors, and inflammation in the hippocampus.

Abbreviations: AHN, adult hippocampal neurogenesis; BDNF, brain-derived neurotrophic factor; BrdU, bromodeoxyuridine; BSA, bovine serum albumin; CA-30, oligosaccharide fraction of LW-AFC; CAT, catalase; DCX, doublecortin; G-CSF, granulocyte colony–stimulating factor; GSH, glutathione; GSH-Px, glutathione peroxidase; HCl, hydrochloric acid; HPA, hypothalamic–pituitary–adrenal axis; HPLC, high-performance liquid chromatography; IGF-1, insulin-like growth factor-1; IL-1β, interleukin-1β; IR, irradiation; IR + LW-AFC, irradiation + LW-AFC; LW, Liuwei Dihuang decoction; LW-AFC, active fraction combination from Liuwei Dihuang decoction; LWB-B, polysaccharide fraction of LW-AFC; LWD-B, glycoside fraction of LW-AFC; MDA, malondialdehyde; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SOD, superoxide dismutase; TCM, traditional Chinese medicine; TNF-α, tumour necrosis factor alpha; VEGF, vascular endothelial growth factor.

Results: LW-AFC exerted beneficial effects on the contextual fear memory, anxiety behaviour, and depression behaviour in irradiated mice. Moreover, LW-AFC increased the number of proliferative neural stem cells and immature neurons in the dorsal hippocampus, displaying a regional specificity of neurogenic response. For the neurogenic microenvironment, LW-AFC significantly increased the contents of superoxide dismutase, glutathione peroxidase, glutathione, and catalase and decreased the content of malondialdehyde in the hippocampus of irradiated mice, accompanied by the increase in brain-derived neurotrophic factor, insulin-like growth factor-1, and interleukin-4 content. Together, LW-AFC improved cognitive and emotional dysfunctions, promoted AHN preferentially in the dorsal hippocampus, and ameliorated disturbance in the neurogenic microenvironment in irradiated mice.

Conclusion: LW-AFC ameliorates cranial irradiation–induced cognitive and emotional dysfunctions, and the underlying mechanisms are mediated by promoting AHN in the dorsal hippocampus and improving the neurogenic microenvironment. LW-AFC might be a promising therapeutic agent to treat cognitive and emotional dysfunctions in patients receiving cranial radiotherapy.

Keywords: traditional Chinese medicine, adult hippocampal neurogenesis, neural stem cells, neurogenic microenvironment, dorsal hippocampus, ventral hippocampus, cranial irradiation, LW-AFC

INTRODUCTION

People are subjected to irradiation exposure commonly during the process of radiodiagnosis and radiotherapy (Brenner and Hall, 2007; Manda and Reiter, 2010; Hladik and Tapio, 2016). A typical example is cranial irradiation which is essential for the treatment of lots of cancer types, such as primary and metastatic brain tumours, and many head and neck malignancies (Monje, 2008). Although cranial irradiation is effective in cancer therapy, it can produce cognitive and emotional dysfunctions which seriously impair the life quality of patients (Son et al., 2015). There are currently no effective clinical interventions for these cognitive and emotional dysfunctions (D'Antonio et al., 2014); therefore, finding therapeutic drugs to ameliorate these cognitive and emotional dysfunctions has become very important.

Cognitive and emotional dysfunctions in patients after treatment with cranial irradiation point to hippocampal damage, and recent evidence indicates that the impairment of adult hippocampal neurogenesis (AHN) is one of the most important mechanisms involved in cranial irradiation–induced cognitive and emotional dysfunctions (Raber et al., 2004; Limoli et al., 2007; Naylor et al., 2008; Acharya et al., 2011). It is accepted that the hippocampus is functionally segregated into the dorsal region mainly implicated in cognitive function and the ventral region crucial for emotional process. In the mammalian brain, AHN occurs in the dentate gyrus of the hippocampus along the dorsal–ventral axis that continuously results in the generation of newborn neurons. AHN is a unique form of structural and functional plasticity and plays an important role in both cognition and emotion (Ming and Song, 2005; Imayoshi et al., 2008; Balu and Lucki, 2009). However, AHN can be severely impaired by cranial irradiation even at low doses, indicating particular vulnerability (Tada et al., 2000; Monje et al., 2002; Mizumatsu et al., 2003). It is also reported that the neurogenic microenvironment (neurogenic niche) in the hippocampus modulates the different processes of neural stem cell development and cranial irradiation damages the neurogenic microenvironment, which mainly correlates with the reduced AHN (Monje and Palmer, 2003; Fike et al., 2007; Ming and Song, 2011; Huang T.-T. et al., 2012). The extreme radiosensitivity of neural stem cells and alterations in the neurogenic microenvironment constitute critical factors in the cognitive and emotional dysfunctions following cranial irradiation. It seems probable that successful interventions for these cognitive and emotional dysfunctions will involve both protection of AHN and drug-based regulation of the neurogenic microenvironment (Monje et al., 2002; Jenrow et al., 2011).

Many traditional Chinese medicine (TCM) prescriptions "nourishing" in the TCM theory system have the effects of improving cognition and emotion (Nishiyama et al., 1994; Itoh et al., 1998; Lin et al., 2003; Yang et al., 2006; Zhang et al., 2016; Hu et al., 2020; Shang et al., 2020). Furthermore, TCM shows good prospects in promoting AHN (Zhang et al., 2014; Sreenivasmurthy et al., 2017; Chen et al., 2019). Liuwei Dihuang decoction (LW), a classical TCM prescription, has been used for the treatment of various diseases with features of "kidney yin" deficiency for about 900 years in China (Huang Y. et al., 2012; Chen et al., 2021). LW consists of six traditional Chinese herbs, *Rehmannia glutinosa* (Gaertn.) DC. [Orobanchaceae; Rehmanniae radix], *Dioscorea oppositifolia* L. [Dioscoreaceae; Dioscoreae rhizoma], *Cornus officinalis* Siebold and Zucc. [Cornaceae; Corni fructus], *Alisma plantago-aquatica* L. [Alismataceae; *Alismatis* rhizoma], *Wolfiporia extensa* (Peck) Ginns (syn. *Poria cocos* (Schw.)).Wolf [Polyporaceae; Poria], and *Paeonia* × *suffruticosa* Andrews [Paeoniaceae; Moutan cortex],

mixed in a ratio of 8:4:4:3:3:3 (Zhou W. et al., 2016). LW is included in the Chinese Pharmacopoeia (2010 version). In the clinic, LW has been used to prevent and treat cognitive dysfunction, depression, climacteric syndrome, cancer, diabetes, and cardiovascular disease (Cheng et al., 2019). A previous study reported that LW could promote AHN in adult rats (Lee et al., 2005). LW-AFC is a new formula of the main active fraction combination extracted from LW. There are three active components in LW-AFC, including polysaccharide fraction (LWB-B), glycoside fraction (LWD-B), and oligosaccharide fraction (CA-30) (Wang et al., 2017a). Previous studies showed that LW-AFC facilitated learning and memory, reduced the amyloid-β (Aβ) plaque load, and restored the imbalance of the hypothalamic–pituitary–adrenal (HPA) axis in the mice model of Alzheimer's disease (Wang et al., 2016; Wang et al., 2017b). LW-AFC also ameliorated long-term potentiation (LTP) impairment in mice induced by acute stress or lipopolysaccharide (Zeng et al., 2019b; Huang et al., 2019). Moreover, LW-AFC was found to enhance learning and memory performance and reduce anxiety- and depression-like behaviour in mice induced by chronic stress (Shen et al., 2018; Zhu et al., 2018; Zeng et al., 2019a). It showed that LW-AFC improved cognitive and emotional dysfunctions in many animal models; however, whether LW-AFC possesses therapeutic effects on cognitive and emotional dysfunctions induced by cranial irradiation has not been reported. Meanwhile, it is not clear whether LW-AFC has beneficial effects on cranial irradiation–induced impairments in AHN along the dorsal–ventral axis and in the neurogenic microenvironment.

The present study aimed at investigating the protective effects of LW-AFC on cognitive and emotional dysfunctions in cranially irradiated mice and whether the underlying mechanisms were mediated by promoting AHN along the dorsal–ventral axis of the hippocampus and ameliorating the neurogenic microenvironment. Given that the dorsal hippocampus is mainly implicated in cognitive function and the ventral hippocampus is predominantly involved in emotional regulation, AHN is not regulated uniformly along the dorsal–ventral axis depending on the stimulus presented (Felice et al., 2012; O'Leary et al., 2012; Vivar et al., 2016). Therefore, in this study, we detected the effect of LW-AFC on AHN in both the dorsal and ventral hippocampus of cranially irradiated mice. Based on the view of regulating AHN and the neurogenic microenvironment, this study should offer a potentially effective treatment for cranial irradiation–induced cognitive and emotional dysfunctions from the resource of TCM and provide scientific evidence for facilitating its application in the clinic. In addition, this study developed a scientific method to investigate whether TCM and its bioactive components could promote AHN accurately in different subregions of the hippocampus along the dorsal–ventral axis.

MATERIALS AND METHODS

Animals

Male C_{57}BL/6J mice (20–22 g, 8 weeks old) were acquired from Beijing Sibeifu Animal Company (Animal Licence No. SCXK 2016-0002; Beijing, China). The mice were housed in groups (4 per cage) and kept under the standard condition (12/12 h light/dark cycle, 22 ± 1°C, food and water ad libitum). All mice adapted to ambient rearing conditions for 7 days before the experiments. All efforts were made to ensure the respect and comfort of the animals. All of the animal's care and treatment, feeding management, and experimental protocols were approved by Institute Animal Care and Use Committee (IACUC) of the National Beijing Center for Drug Safety Evaluation and Research (NBCDSER) (No. 2018-030), in compliance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996).

Drugs and Reagents

Bromodeoxyuridine (BrdU) (B5002), Triton X-100 (T8787), and bovine serum albumin (BSA) (A3858) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Paraformaldehyde (CAS: 30525-89-4), sucrose (CAS: 57-50-1), hydrochloric acid (HCl) (CAS: 7647-01-0), boric acid (CAS: 10043-35-3), and alcohol (CAS: 64-17-5) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Sodium pentobarbital (Lot. 34-06-12) was purchased from Shanghai Chemical Reagent Assembly Factory (Shanghai, China). These drugs and reagents were all of analytically pure grade.

Anti-BrdU antibody (ab6326) was obtained from Abcam (Cambridge, United Kingdom), and anti-doublecortin (DCX) antibody (#4604) was purchased from Cell Signaling Technology (Boston, MA, United States). Alexa Fluor 488-conjugated donkey anti-rat IgG (A21208) and Alexa Fluor 594-conjugated goat anti-rabbit IgG (A11037) were acquired from Thermo Fisher Scientific (Waltham, MA, United States). The fluorescent mounting medium containing DAPI (ZLI-9557) was purchased from Zhongshan Jinqiao Biotechnology Co., Ltd. (Beijing, China). Phosphate-buffered saline (PBS) (AR0030) was obtained from Boster Biological Technology Co., Ltd. (Wuhan, China). Normal saline (Lot. 1811242004) was purchased from Shijiazhuang Siyao Co., Ltd. (Shijiazhuang, China).

The Preparation of LW-AFC

The original herbs of Liuwei Dihuang decoction (LW) were purchased from Beijing Tongrentang Pharmacy (Beijing, China), which included 32% *Rehmannia glutinosa* (Gaertn.) DC. [Orobanchaceae; Rehmanniae radix] (Lot. 20150130), 16% *Dioscorea oppositifolia* L. [Dioscoreaceae; Dioscoreae rhizoma] (Lot. 1503028), 16% *Cornus officinalis* Siebold and Zucc. [Cornaceae; Corni fructus] (Lot. 20151126), 12% *Alisma plantago-aquatica* L. [Alismataceae; Alismatis rhizoma] (Lot. 20160116), 12% *Wolfiporia extensa* (Peck) Ginns (syn. *Poria cocos* (Schw.)). Wolf [Polyporaceae; Poria] (Lot. 1601001), and 12% *Paeonia* × *suffruticosa* Andrews [Paeoniaceae; Moutan cortex] (Lot. 20160526) at a proportion of 8:4:4:3:3:3. The total dry weight was 3 kg. They were authenticated by Professor Yimin Zhao and Shanyi Qiao (Department of Phytochemistry, Beijing Institute of Pharmacology and Toxicology) according to Chinese Pharmacopoeia (2010 version). The voucher specimens were stored at the Department of Phytochemistry, Beijing Institute of Pharmacology and Toxicology.

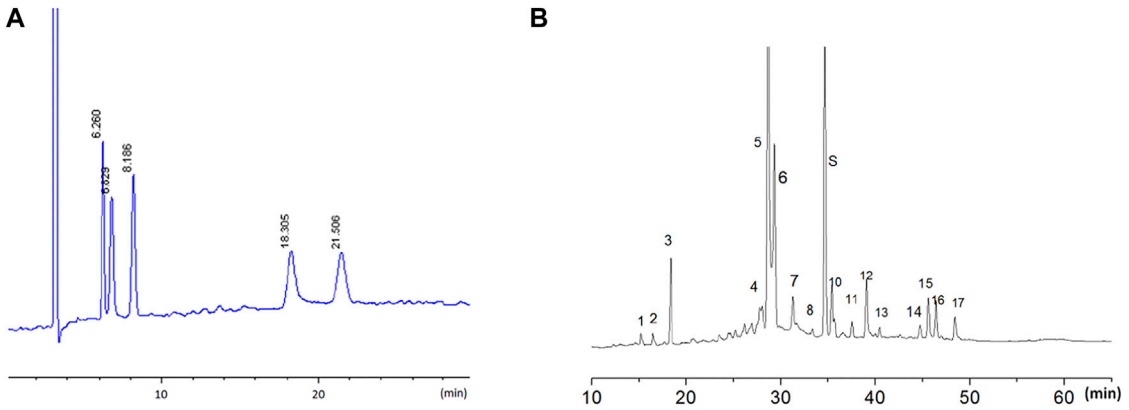

FIGURE 1 | The HPLC fingerprint of LW-AFC. LW-AFC consists of polysaccharide fraction (LWB-B), glycoside fraction (LWD-B), and CA-30. **(A)** The HPLC fingerprint of LWB-B and CA30 mixture. The peak at 6.26, 6.83, 8.19, 18.30, and 21.50 min represents, respectively, fructose, glucose, sucrose, mannotriose, and stachyose. **(B)** The HPLC fingerprint of LWD-B. The peak S represents loganin.

LW-AFC was prepared from LW. The details are displayed in **Supplementary Materials 2**. We briefly describe the method of preparation here. Six herbs of LW, including *Rehmannia glutinosa* (Gaertn.) DC. [Orobanchaceae; Rehmanniae radix], *Dioscorea oppositifolia* L. [Dioscoreaceae; Dioscoreae rhizoma], *Cornus officinalis* Siebold & Zucc. [Cornaceae; Corni fructus], *Alisma plantago-aquatica* L. [Alismataceae; Alismatis rhizoma], *Wolfiporia extensa* (Peck) Ginns (syn. *Poria cocos* (Schw.)). Wolf [Polyporaceae; Poria], and *Paeonia* × *suffruticosa* Andrews [Paeoniaceae; Moutan cortex], were mixed according to the dry weight ratio of 8:4:4:3:3:3. The mixture of herb materials was decocted with 10 volumes of deionized water with boiling refluxing thrice, 2 h each time. After finishing the extraction, the materials were filtered through a 6-layer gauze to yield three extraction solutions at 50°C, allowed to cool to room temperature, and centrifuged (2500 rpm/min, 25 min). The supernatant filtered from LW was concentrated into a quintessence. The quintessence was then extracted using ethanol to produce the supernatant (LWD), and the sediment left in the deionized water was concentrated into the dried LWB-B. LWD was concentrated, and ethanol was removed. LWD was then dissolved in deionized water and eluted in turn with deionized water and 30% ethanol on microporous adsorptive resins. The 30% ethanol elution of LWD was cryodesiccated into LWD-B, and the water elution of LWD was concentrated and eluted in turn with 5% ethanol and 30% ethanol on an active carbon absorption column. The 30% ethanol elution was then concentrated, ethanol was removed, and the elution was cryodesiccated into the CA-30.

The HPLC Fingerprint for Quality Control of LW-AFC

LW-AFC includes 20.3% LWB-B, 15.1% LWD-B, and 64.6% CA-30 at a dry weight ratio. LWD-B mainly contains loganin, loganic acid, morroniside, sweroside, paeoniflorin, acteoside, isoacteoside, jionoside A_1, jionoside A_2, jionoside B_1, jionoside B_2, and 5-hydroxymethyl-furaldehyde. CA-30 mainly contains mannotriose and stachyose. LWB-B is mainly composed of polygalacturonic acid, rhamnogalacturonic acid polysaccharide, arabinogalactan, and dextran (Cheng et al., 2019).

The LW-AFC components were detected using the high-performance liquid chromatography (HPLC) method for quality control. The HPLC fingerprint of LW-AFC is shown in **Figure 1**. In brief, for LWB-B and CA30 mixture, the chromatographic separation was obtained on a NucleosilNH2 100 Å column; five chromatogram peaks were observed, representing fructose, glucose, sucrose, mannotriose, and stachyose. The retention time of these peaks was 6.260, 6.829, 8.186, 18.305, and 21.506 min, respectively (**Figure 1A**). For LWD-B, chromatographic separation was obtained on a Diamond C18 column; there were 17 chromatogram peaks in the fingerprint of LWD-B. The S peak represented loganin (**Figure 1B**).

Experiment Design

Male C_{57}BL/6J mice of 8 weeks old were randomly divided into three groups: control group, irradiation group, and irradiation + LW-AFC group (IR + LW-AFC). Each group had 24 mice. Before irradiation, the mice of the IR + LW-AFC group were given an intragastric administration of LW-AFC (1.6 g/kg body weight) for 2 weeks, while the mice in the control and IR group were given the same volume of distilled water. Next, the mice in the IR and IR + LW-AFC group received 7 Gy γ-ray cranial irradiation, while the mice of the control group were placed into the irradiation device without irradiation. Two days before irradiation, the contextual fear conditioning test was performed, until the end of the experiment. On the first day after irradiation, the open field test and tail suspension test were performed. When the behavioural tests were completed, half of the mice in each group were transcardially perfused and the brains of mice were removed for immunofluorescent staining. In addition, the other half of mice in each group were sacrificed for the multiplex bead analysis and assay of oxidative stress level (**Figure 2A**).

LW-AFC is a new formula of the main active fraction combination extracted from LW. From the previous experiments in our laboratory, we have demonstrated that LW-AFC (1.6 g/kg) is the optimal resource in improving cognitive and emotional

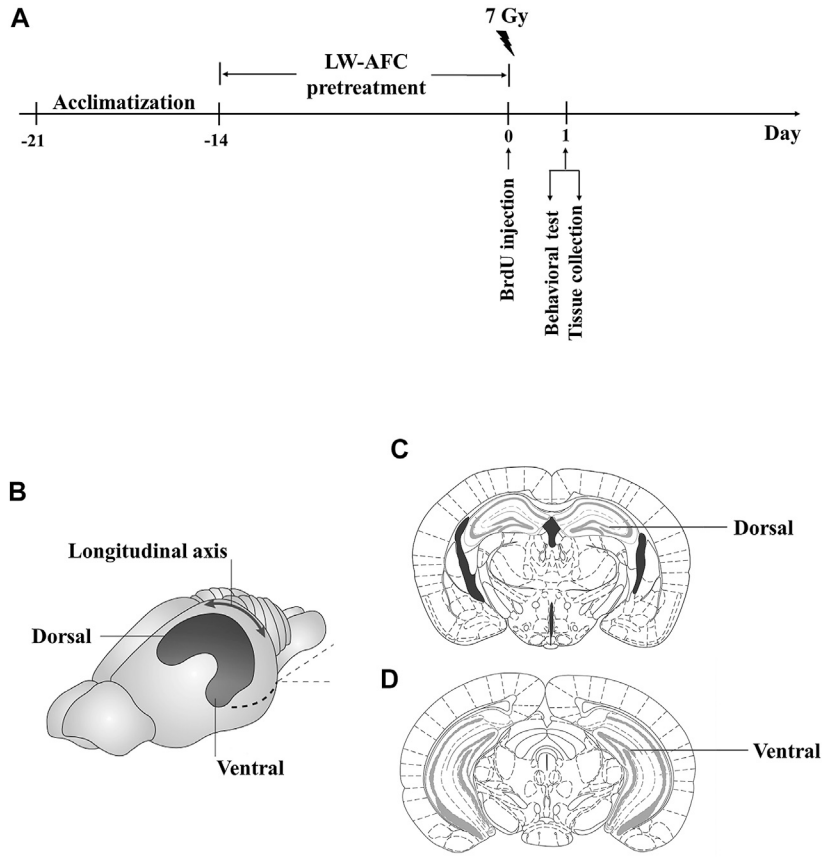

FIGURE 2 | Scheme of the experimental procedure and schematic representation of the dorsal and ventral hippocampus in mice. **(A)** Scheme of the experimental procedure. **(B)** The image of the dorsal and ventral hippocampus along the longitudinal axis. **(C)** The coronal brain section of the dorsal hippocampus. **(D)** The coronal brain section of the ventral hippocampus.

dysfunctions in many animal models (Wang et al., 2016; Wang et al., 2017b; Shen et al., 2018; Zhu et al., 2018; Zeng et al., 2019b; Huang et al., 2019). Therefore, we selected the dose of LW-AFC as 1.6 g/kg in this study.

Preparation of the Mice Model of Cranial Irradiation

For cranial irradiation, mice were anaesthetised by intraperitoneal injection of 1% sodium pentobarbital in normal saline (0.05 ml/10 g body weight), covered with a sliding shield which protected the body of mice and exposed only the hippocampus to irradiation, and finally irradiated with γ-ray at a single dose of 7 Gy. After the irradiation was completed, the mice were placed into a box containing cotton around the electric heater for rising the temperature quickly so that they wake up as soon as possible. The mice were returned to the home cage for further rearing.

Bromodeoxyuridine Treatment

After water bath at 37°C for 30 min, BrdU was dissolved in normal saline at a concentration of 2 mg/ml. To test the cell proliferation, mice of all groups received intraperitoneal injection of BrdU solution (50 mg/kg body weight, twice a day, 6 h interval) after irradiation exposure and were perfused for 16 h after the last BrdU injection.

Preparation of Brain Sections in the Dorsal and Ventral Hippocampus

After perfusion, mice brains were fixed in 4% paraformaldehyde in PBS (0.1 M, pH 7.4) at 4°C for 12 h and dehydrated with 30% sucrose in PBS for 3 days. 40 um-thick sections were cut with a freezing microtome (CM 1950, Leica, Germany) and stored in PBS at 4°C before use. The sections from the dorsal and ventral hippocampus were used for immunofluorescent staining. The dorsal hippocampus and the ventral hippocampus were dissociated according to coordinates: −0.94 to −2.30 mm relative to the bregma for the dorsal hippocampus and −2.46 to −3.80 mm for the ventral hippocampus (O'Leary et al., 2012; Zheng et al., 2017). Five sections were chosen for dorsal hippocampus or ventral hippocampus, respectively.

Immunofluorescent Staining

The proliferation of neural stem cells and the level of immature neurons are the sensitive indicators for detecting AHN, which can be evaluated by BrdU-positive cells and DCX-positive cells with

immunofluorescent staining. For BrdU-positive cell detection, the sections were washed three times in PBS first. Then, the sections were treated with 2M HCl for 1 h at room temperature to denature DNA followed by immersion in 0.1 M boric acid (PH 8.5) for 10 min at room temperature, rinsed three times in PBS, blocked in 0.1% Triton X-100 and 3% BSA in PBS for 1 h, and incubated with rat anti-BrdU antibody (1:200) in PBS with 0.1% Triton X-100 and 3% BSA overnight at 4°C. The next day, after washing in PBS, sections were incubated with Alexa Fluor 488-conjugated donkey anti-rat IgG (1:1,000) in PBS with 0.1%Triton X-100 and 3% BSA for 1 h at room temperature. After washing in PBS again, the sections were mounted on slides with the fluorescent mounting medium containing DAPI, covered with coverslips, and stored at −20°C for further examination.

For DCX-positive cell detection, the sections were washed three times in PBS first. Next, the sections were treated with 0.5%Triton X-100 for 30 min at room temperature, blocked in 0.1% Triton X-100 and 3% BSA in PBS for 1 h, and incubated with rabbit anti-DCX antibody (1:400) in PBS with 0.1%Triton X-100 and 3% BSA overnight at 4°C. The next day, after washing in PBS, sections were incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:1,000) in PBS with 0.1%Triton X-100 and 3% BSA for 1 h at room temperature. After washing in PBS again, the sections were mounted on slides with the fluorescent mounting medium containing DAPI, covered with coverslips, and stored at −20°C for further examination. BrdU-positive cells and DCX-positive cells were visualized and counted using a Zeiss 880 confocal microscope (Zeiss, Oberkochen, Germany).

Measurement of Inflammatory, Neurotrophic, and Growth Factors

The proinflammatory factors to be detected include interleukin-1β (IL-1β), IL-6, IL-17A, and tumour necrosis factor alpha (TNF-α). Anti-inflammatory factors include IL-4, IL-10, granulocyte colony–stimulating factor (G-CSF). Neurotrophic and growth factors include brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor (VEGF). All of them were detected by multiplex bead analysis as previously described (Wang et al., 2016). The samples of the hippocampus were analysed using Luminex 200™ (Luminex, TX, United States). The levels of these factors were measured using a multifactor detection kit (LXSAMSM-11, R&D Systems, United States) according to the manufacturer's instructions.

Assay of the Oxidative Stress Level

The indexes of oxidative stress include glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT), superoxide dismutase (SOD), and malondialdehyde (MDA). These indexes were detected by colorimetry. After hippocampus tissues were homogenized and centrifuged, the supernatant was collected and measured using specific commercial kits related to these indexes (A006-1-1, A005-1-2, A007-1-1, A001-1-1, and A003-1-1; Nanjing Jiancheng Institute of Biological Engineering, China) according to the manufacturer's instructions.

Open Field Test

The open field box is made of black polyvinyl chloride (420 × 420 × 420 mm), and there is a center zone in the middle of the box with a permanent marker (205 × 205 mm). The mice were handled every day to be familiar with the experimenter before all behavioural tests. Before the open field test, the mice adapted to the environment of the experimental room for 1 h first. Next, the mice were put into the box of open field gently and explored freely for 5 min. The trajectories of mice were recorded by ANY-maze software (Stoelting Co., United States). When the mice were taken out, the box was wiped with 75% alcohol to remove the odour before the next mice to be tested. ANY-maze software recorded the time of mice in the center zone of the open field within 5 min.

Contextual Fear Conditioning Test

The contextual fear conditioning test was conducted in chambers with internal dimensions of 30 cm width × 30 cm length × 60 cm height. A house light above the chamber provided illumination. The mice were placed in the environment of the experimental room to adapt for 1 h before the test. The experiment lasted 3 days. The first day was the adaptation period, and the mice were gently placed in the experiment chamber to explore freely for 30 min. The second day was the learning period, and the mice were shocked to absorb the harmful stimulus in the chamber for 5 min. The current parameters were 0.8 mA and 5 times/300 s, the interval time for shock was 60 s, and the duration time for shock was 2 s. The third day was the test period. All the contexts in the experiment chamber remained unchanged. The mice were gently put into the experiment chamber, and the freezing time of mice was recorded within 5 min.

Tail Suspension Test

The mice accommodated the environment of the experimental room for 1 h before the test. First, the tails of mice were stuck to one side of the adhesive plaster, and the other side of the adhesive plaster was stuck to the hooks of the experiment box as soon as possible. When all mice were suspended to the hangers, the ANY-maze software recorded the trace of mice for 6 min. The immobility time of mice in the latter 4 min in the experiment box was needed for statistical analysis.

Statistical Analysis

Data were expressed as mean ± standard deviation (S.D.). Data were analysed using the unpaired Student's t test and one-way analysis of variance (ANOVA) with the appropriate post hoc test for multiple comparisons where appropriate. The value of $p < 0.05$ was considered as statistically significant.

RESULTS

LW-AFC Pretreatment Improved Cognitive and Emotional Dysfunctions in Cranially Irradiated Mice

To observe the effects of LW-AFC pretreatment for 2 weeks on cognitive and emotional dysfunctions induced by cranial

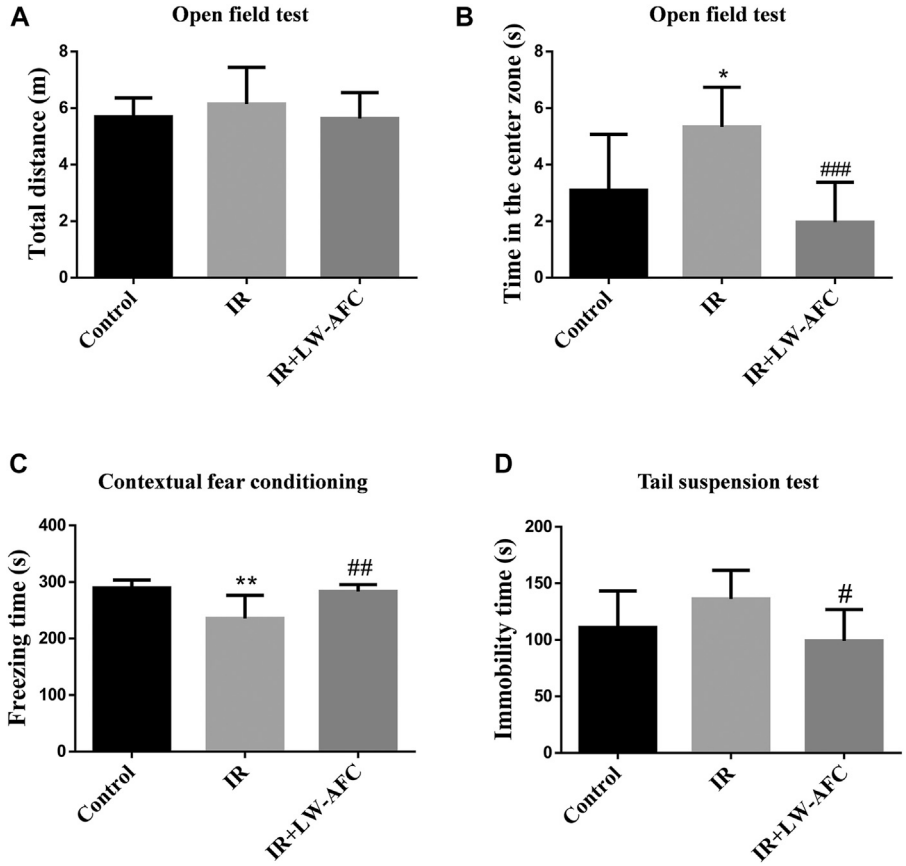

FIGURE 3 | LW-AFC improves cognitive and emotional dysfunctions in IR mice on day 1 after irradiation. **(A)** Quantification of the total distances travelled by mice during the open field test. **(B)** Quantification of the center time of mice during the open field test. **(C)** Quantification of the freezing time of mice during the contextual fear conditioning test. **(D)** Quantification of the immobility time of mice during the tail suspension test. The values denote mean ± S.D., $n = 8$. $^{*}p < 0.05$ and $^{**}p < 0.01$, versus the control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$, versus the IR group. Abbreviation: IR, irradiation.

irradiation, the contextual fear conditioning, open field, and tail suspension tests were used on the first day after irradiation.

In the open field test, there was no difference in the total distances travelled by mice among the three groups, suggesting that the locomotor activity of mice was not changed (**Figure 3A**). A significant increase in the time in the center zone was observed in IR mice compared with the control mice, and this change was restored by LW-AFC pretreatment ($p < 0.001$) (**Figure 3B**). The results revealed that cranial irradiation reduced the anxiety level in mice and LW-AFC reversed cranial irradiation–induced reduction in the anxiety level.

In the contextual fear conditioning test, compared with the control mice, the freezing time of IR mice was significantly reduced and this change could be reversed by LW-AFC pretreatment ($p < 0.01$) (**Figure 3C**). The results demonstrated that cranial irradiation impaired the contextual fear memory in mice and LW-AFC significantly improved cranial irradiation–induced impairment in the contextual fear memory.

In the tail suspension test, compared with the control mice, the immobility time in IR mice displayed an increasing tendency. The immobility time in IR mice pretreated with LW-AFC was significantly decreased compared with the IR mice ($p < 0.05$) (**Figure 3D**). The results suggested that cranial irradiation tended to induce depression-like behaviour in mice and LW-AFC reduced depression-like behaviour in IR mice.

Collectively, cranial irradiation led to cognitive and emotional dysfunctions in mice on the first day after irradiation, and these dysfunctions could be ameliorated significantly by LW-AFC.

LW-AFC Pretreatment Increased the Number of Proliferative Neural Stem Cells in the Dorsal Hippocampus of Irradiated Mice

To assess the effect of LW-AFC pretreatment on the proliferation of neural stem cells in the dentate gyrus of the hippocampus along the dorsal–ventral axis in IR mice, the number of BrdU-positive cells visualized by immunofluorescence staining was quantified. BrdU is a thymine nucleoside analogue, which can replace thymine (T) to penetrate into the replicating DNA molecules during cell proliferation (Martel et al., 2016). A schematic representation of the dorsal and ventral hippocampus is exhibited in **Figures 2B–D**. On the first day after irradiation,

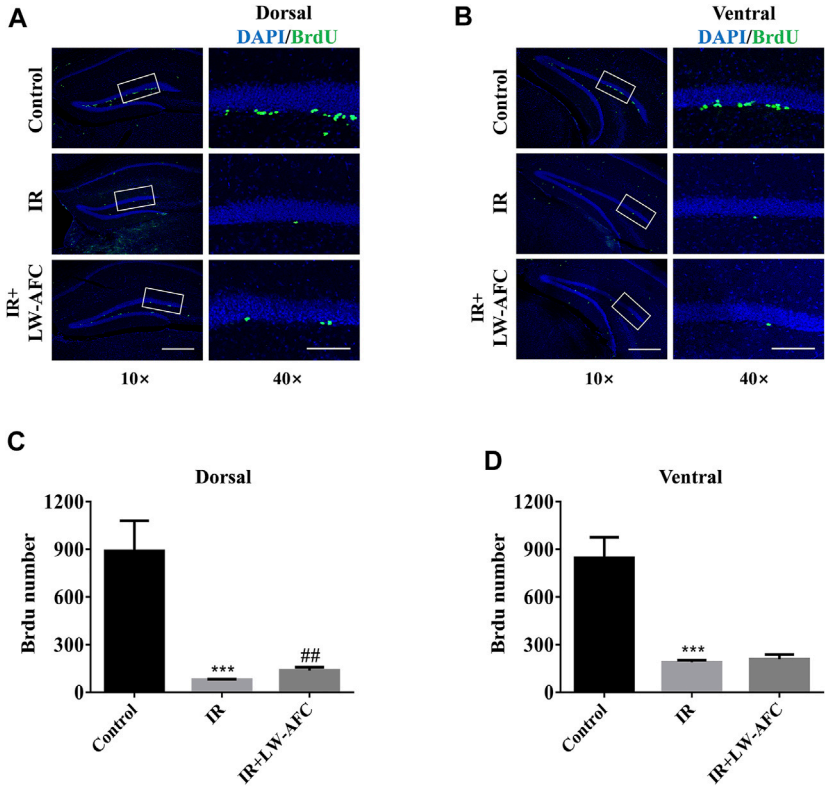

FIGURE 4 | LW-AFC promotes the proliferation of neural stem cells preferentially in the dorsal hippocampus of IR mice on day 1 after irradiation. Representative immunofluorescence images of BrdU-positive cells in the **(A)** dorsal hippocampus and the **(B)** ventral hippocampus. The area of white square frame in the left image indicates the enlarged image of BrdU-positive cells in the right. The confocal images in the left are of 10×, and the enlarged images in the right are of 40×. Quantification of BrdU-positive cells in the **(C)** dorsal hippocampus and the **(D)** ventral hippocampus. The scale bar in the left image = 300 μm, and the scale bar of the enlarged image = 100 μm. The values denote mean ± S.D., $n = 4$. $^{***}p < 0.001$, versus the control group; $^{\#\#}p < 0.01$, versus the IR group. Abbreviation: IR, irradiation.

compared with the control mice, the number of BrdU-positive cells in the dorsal hippocampus of IR mice was significantly decreased and this change could be improved significantly by LW-AFC pretreatment ($p < 0.01$) (**Figures 4A,C**). Compared with the control mice, a significant decrease in the number of BrdU-positive cells in the ventral hippocampus was observed in IR mice; however, the LW-AFC pretreatment did not improve this change (**Figures 4B,D**). The results showed that the proliferation of neural stem cells in the hippocampus along the dorsal–ventral axis was significantly damaged on the first day after irradiation and LW-AFC significantly improved cranial irradiation–induced impairment in the proliferation of neural stem cells in the dorsal hippocampus with no effect in the ventral hippocampus.

LW-AFC Pretreatment Increased the Number of Immature Neurons in the Dorsal Hippocampus of Irradiated Mice

To examine the effect of LW-AFC pretreatment on immature neurons in the dentate gyrus of the hippocampus along the dorsal–ventral axis in IR mice, the number of DCX-positive cells visualized by immunofluorescence staining was quantified. DCX is commonly used as a marker of immature neurons. On the first day after irradiation, compared with the control mice, the number of DCX-positive cells in the dorsal hippocampus of IR mice was significantly reduced and this change could be ameliorated significantly by LW-AFC pretreatment ($p < 0.05$) (**Figures 5A,C**). Compared with the control mice, the number of DCX-positive cells in the ventral hippocampus of IR mice was significantly decreased; however, this decrease could not be attenuated by the LW-AFC pretreatment (**Figures 5B,D**). The results showed that cranial irradiation reduced the number of immature neurons in both the dorsal and ventral hippocampus on the first day after irradiation. LW-AFC ameliorated cranial irradiation–induced reduction in the number of immature neurons in the dorsal hippocampus but had no effect on the ventral hippocampus.

Combined with the experimental data about the effect of LW-AFC on the proliferation of neural stem cells, we concluded that AHN was significantly damaged along the dorsal–ventral axis on the first day after irradiation and LW-AFC improved the impaired AHN predominantly in the dorsal hippocampus, which might be related to the improvement of behavioural dysfunctions.

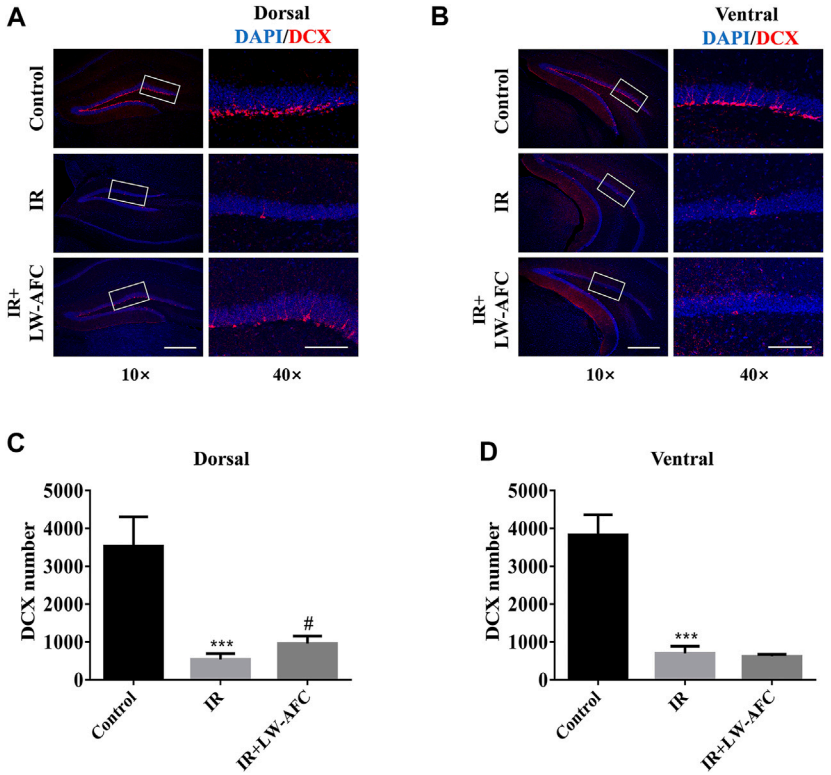

FIGURE 5 | LW-AFC increases the number of immature neurons preferentially in the dorsal hippocampus of IR mice on day 1 after irradiation. Representative immunofluorescence images of DCX-positive cells in the **(A)** dorsal hippocampus and **(B)** ventral hippocampus. The area of white square frame in the left image indicates the enlarged image of DCX-positive cells in the right. The confocal images in the left are of 10×, and the enlarged images in the right are of 40×. Quantification of DCX-positive cells in the **(C)** dorsal hippocampus and the **(D)** ventral hippocampus. The scale bar in the left image = 300 μm, and the scale bar of the enlarged image = 100 μm. The values denote mean ± S.D., $n = 4$. $^{***}p < 0.001$, versus the control group; $^{\#}p < 0.05$, versus the IR group. Abbreviation: IR, irradiation.

LW-AFC Pretreatment Ameliorated the Neurogenic Microenvironment in the Hippocampus of Irradiated Mice

The neurogenic microenvironment regulates the development and fate of neural stem cells, but cranial irradiation can lead to the alterations in the neurogenic microenvironment, which significantly contributes to the AHN impairment and cognitive and emotional dysfunctions from the cranial irradiation (Monje et al., 2002; Monje and Palmer, 2003; Jenrow et al., 2011; Ming and Song, 2011). Therefore, we further investigated whether LW-AFC pretreatment improved the neurogenic microenvironment in the hippocampus of IR mice on the first day after irradiation. We focused on the changes in the level of oxidative stress, neurotrophic and growth factors, and inflammation in the hippocampus of mice among three groups.

The Effect of LW-AFC Pretreatment on the Level of Oxidative Stress in the Hippocampus of Irradiated Mice

To evaluate the effect of LW-AFC pretreatment on the level of oxidative stress in the hippocampus of IR mice, the contents of SOD, GSH-Px, GSH, CAT, and MDA were detected. The contents of SOD, GSH-Px, and CAT were reduced significantly, the GSH content tended to decrease, and the MDA content tended to increase in the hippocampus of IR mice, compared with the control mice. The LW-AFC pretreatment significantly increased the contents of SOD ($p < 0.001$), GSH-Px ($p < 0.001$), GSH ($p < 0.01$), and CAT ($p < 0.001$) and significantly reduced the content of MDA ($p < 0.05$) in the hippocampus of IR mice (**Figure 6**). The results showed that cranial irradiation increased the level of oxidative stress in the hippocampus of mice and this change could be reversed by LW-AFC.

The Effect of LW-AFC Pretreatment on the Level of Neurotrophic and Growth Factors in Hippocampus of Irradiated Mice

To assess the effect of LW-AFC pretreatment on the level of neurotrophic and growth factors in the hippocampus of IR mice, the contents of BDNF, IGF-1, and VEGF were examined. The contents of BDNF, IGF-1, and VEGF were significantly decreased in the hippocampus of IR mice compared with the control mice. The contents of BDNF ($p < 0.05$) and IGF-1 ($p < 0.05$) were significantly increased in the hippocampus of IR mice by LW-AFC pretreatment (**Figure 7**). The results suggested that cranial irradiation reduced the level of neurotrophic and growth

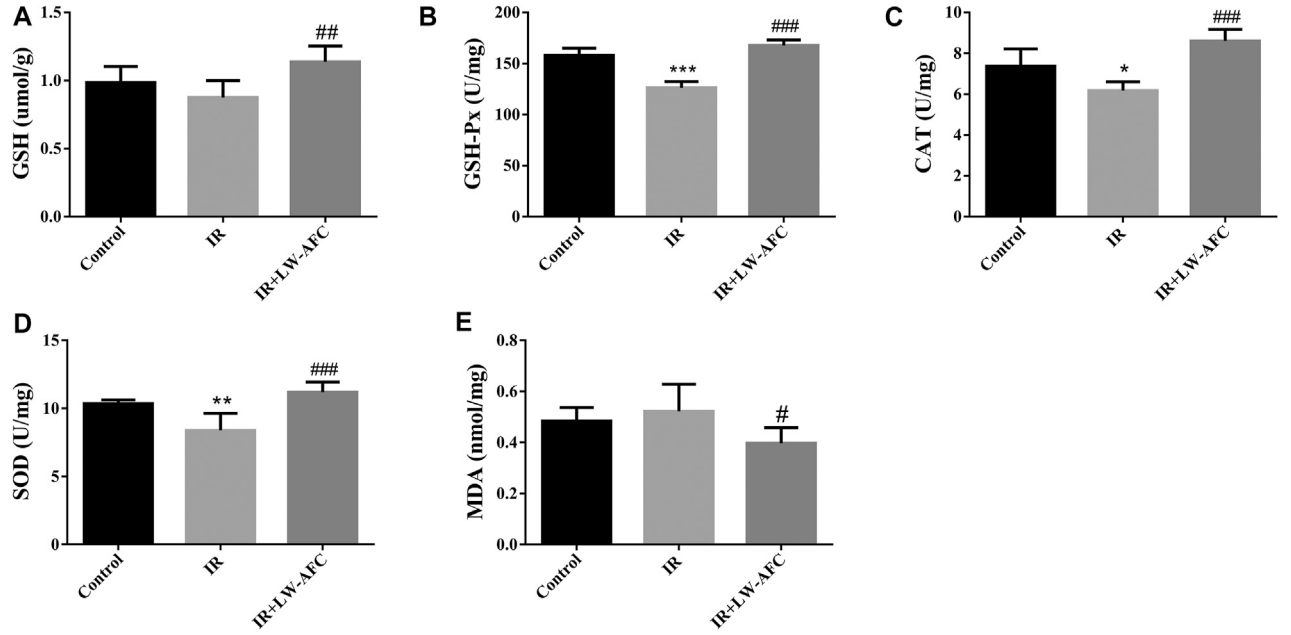

FIGURE 6 | LW-AFC reduces the oxidative stress level in the hippocampus of IR mice on day 1 after irradiation. Concentrations of **(A)** GSH, **(B)** GSH-Px, **(C)** CAT, **(D)** SOD, and **(E)** MDA were detected. The values denote mean ± S.D., $n = 6$. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, versus the control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$, versus the IR group. Abbreviation: IR, irradiation.

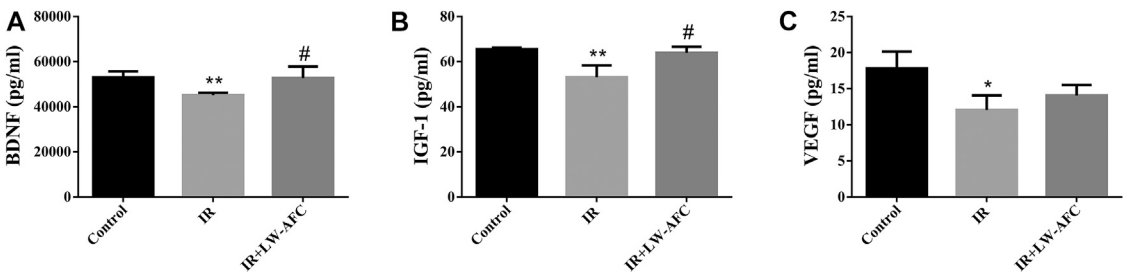

FIGURE 7 | LW-AFC increases the level of neurotrophic and growth factors in the hippocampus of IR mice on day 1 after irradiation. Concentrations of **(A)** BDNF, **(B)** IGF-1, and **(C)** VEGF were detected. The values denote mean ± S.D., $n = 4$. $^{*}p < 0.05$ and $^{**}p < 0.01$, versus the control group. $^{\#}p < 0.05$, versus the IR group. Abbreviation: IR, irradiation.

factors in the hippocampus of mice and this reduction could be mitigated by LW-AFC.

The Effect of LW-AFC Pretreatment on the Level of Inflammation in the Hippocampus of Irradiated Mice

To observe the effect of LW-AFC pretreatment on the level of inflammation in the hippocampus of IR mice, the proinflammatory factors and anti-inflammatory factors were detected. The proinflammatory factors included IL-1β, IL-6, IL-17A, and TNF-α. The anti-inflammatory factors included IL-4, IL-10, and G-CSF. The contents of IL-6, TNF-α, and IL-17A were significantly increased (**Supplementary Figure S1**) and the content of IL-4 was significantly decreased in the hippocampus of IR mice, compared with the control mice (**Figure 8A**). LW-AFC pretreatment significantly increased the content of IL-4 ($p < 0.01$) in the hippocampus of IR mice. The results demonstrated that cranial irradiation elevated the level of inflammation in the hippocampus of mice and this elevation could be attenuated by LW-AFC through modulating the anti-inflammatory factor.

Together, our study indicated that cranial irradiation disrupted the neurogenic microenvironment in the hippocampus of mice and LW-AFC ameliorated cranial irradiation–induced disturbance in the neurogenic microenvironment characterized by decrease in the level of oxidative stress and increase in the level of neurotrophic and growth factors as well as the anti-inflammatory factor, which were associated with LW-AFC–induced promotion of AHN and protection against cognitive and emotional dysfunctions.

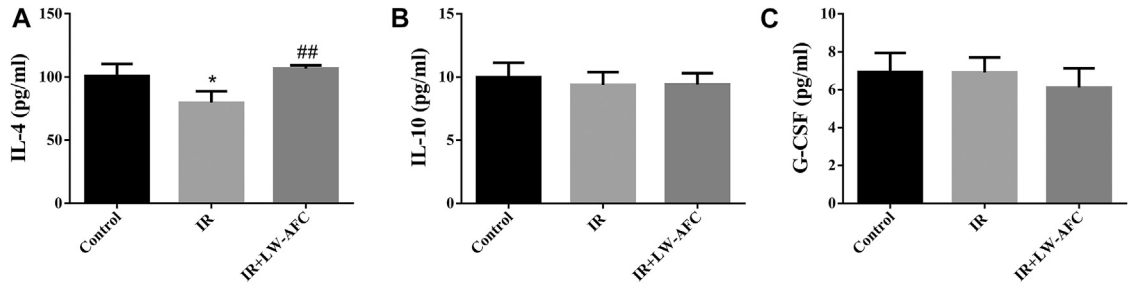

FIGURE 8 | LW-AFC increases the level of anti-inflammatory factor in the hippocampus of IR mice on day 1 after irradiation. Concentrations of **(A)** IL-4, **(B)** IL-10, and **(C)** G-CSF were detected. The values denote mean ± S.D., $n = 4$. $^{*}p < 0.05$, versus the control group; $^{\#\#}p < 0.01$, versus the IR group. Abbreviation: IR, irradiation.

DISCUSSION

Cranial radiotherapy is a necessary strategy in the treatment of primary and metastatic brain tumours, but it also leads to cognitive and emotional deficits, which are closely related to hippocampal dysfunctions. Some reports suggest that cranial irradiation–induced cognitive and emotional deficits are significantly linked to reduced AHN in the dentate gyrus of the hippocampus (Raber et al., 2004; Winocur et al., 2006). Moreover, cranial irradiation causes disturbance in the neurogenic microenvironment, which damages the neurogenic potential of the hippocampus and potentially contributes to these cognitive and emotional deficits (Monje et al., 2002; Rola et al., 2004). Promoting endogenous AHN and regulating the neurogenic microenvironment represent a new strategy for ameliorating cranial irradiation–induced functional deficits. In this study, we found that LW-AFC, when given 2 weeks before 7 Gy γ-ray cranial irradiation, could significantly improve cognitive and emotional deficits after cranial irradiation, and this effect was mediated by promoting AHN preferentially in the dorsal hippocampus and ameliorating the neurogenic microenvironment of the hippocampus in mice. It suggested that LW-AFC protected against the adverse effects and aided the recovery of hippocampal function following cranial irradiation.

Lots of studies have shown that the hippocampus is a structure with different anatomical connections, molecular features, and functions along the dorsal–ventral axis (Thierry et al., 2000; Kishi et al., 2006; Cenquizca and Swanson, 2007; Tanti et al., 2012). The dorsal hippocampus is mainly implicated in cognitive function, and the ventral hippocampus mainly mediates affective processes (Moser and Moser, 1998; Bannerman et al., 2004). AHN is a special neuroplasticity that produces the newborn neurons in the dentate gyrus of the hippocampus throughout the life of mammals (Gonçalves et al., 2016; Abbott and Nigussie, 2020). It is noted that AHN differs along the dorsal–ventral axis; for example, the dorsal hippocampus displays a faster maturation rate of newborn neurons than the ventral hippocampus (Snyder et al., 2009; Jinno, 2011; Snyder et al., 2012). AHN may also be preferentially affected in either the dorsal hippocampus or ventral hippocampus depending on the exact stimulation of the experiments, which suggests a regional specificity of AHN response; for example, a previous research revealed that environmental enrichment increased AHN in the dorsal hippocampus preferentially (Tanti et al., 2012). Therefore, it is reasonable to consider that the hippocampal function varies along the dorsal–ventral axis when AHN is examined, especially in experiments where drug therapy may affect AHN (O'Leary et al., 2012; Vivar et al., 2016; Zhou Q.-G. et al., 2016).

A previous study showed that the mice with ablation of AHN by cranial irradiation displayed more time in the open arm in the elevated plus maze and exhibited no difference in the immobility time in the forced swimming test, indicating reduced anxiety-like behaviour in neurogenesis-deficient mice (Tsai et al., 2015). Some studies revealed that cranial irradiation induced depression-like behaviour in mice (Wong-Goodrich et al., 2010; Son et al., 2014). Ablation of AHN by cranial irradiation had been previously reported to impair contextual fear conditioning in mice without affecting spatial memory, including the Morris water maze task and place recognition task of Y maze (Saxe et al., 2006). Another research showed that cranial irradiation reduced AHN and impaired performance of the object location task in rats; however, no overall cognitive impairment was observed (Lensu et al., 2020). Differences in studies about how AHN damage affected cognitive and emotional behaviour resulted from multiple factors, such as strain, gender, age of the animals, and experimental procedures.

Our study demonstrated that cranial irradiation decreased the number of proliferating neural stem cells and immature neurons in both the dorsal and ventral hippocampus on the first day after irradiation, suggesting that the AHN of mice was seriously damaged along the dorsal–ventral axis. Our results also revealed that mice with AHN damage exhibited the impairment of contextual fear memory, reduced anxiety level in the open field test, and showed an elevated tendency in the depression level in the tail suspension test on the first day after irradiation. Combined with the data on AHN and behaviour, we concluded that the impairment of AHN in both the dorsal and ventral hippocampus contributed to cognitive and emotional dysfunctions in mice.

There are currently no successful or effective interventions for cranial irradiation–induced cognitive and emotional dysfunctions (D'Antonio et al., 2014). Many TCM prescriptions "nourishing" in the TCM theory system have the

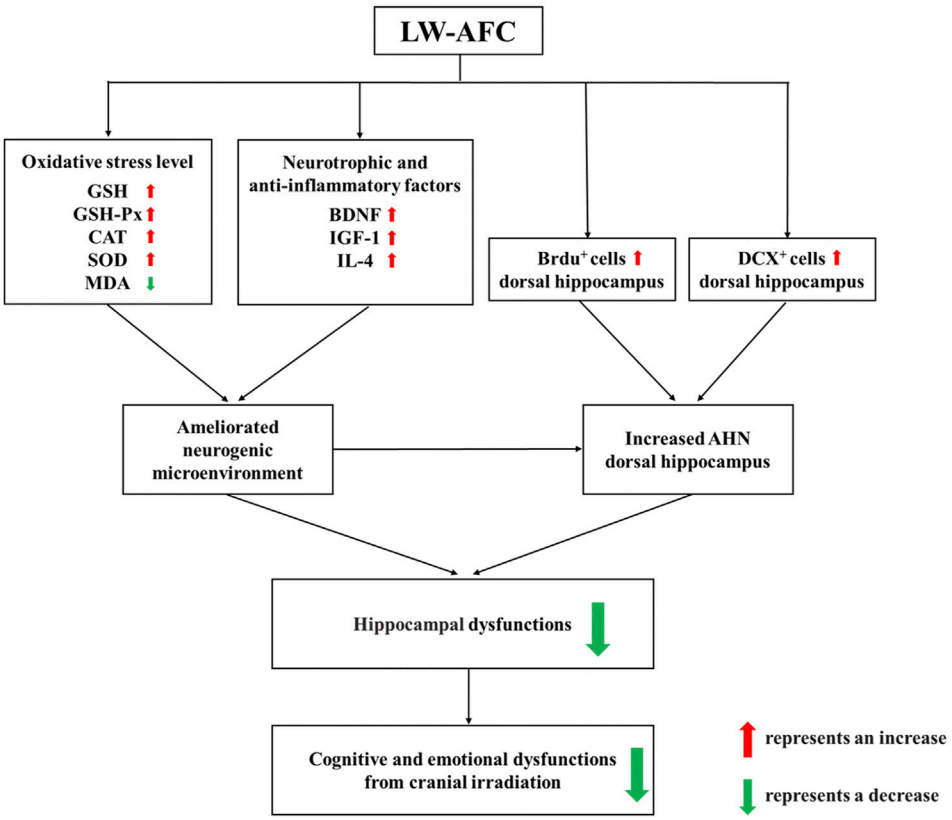

FIGURE 9 | A schematic illustration of the protective mechanisms of LW-AFC against cranial irradiation–induced cognitive and emotional dysfunctions via increasing AHN in the dorsal hippocampus and modulating the neurogenic microenvironment.

effects of improving cognition and emotion and display the ability to promote AHN (Zhang et al., 2014; Sreenivasmurthy et al., 2017). LW, a classical TCM prescription, has been used clinically in the treatment of various diseases with signs of "kidney yin" deficiency (Hsieh et al., 2003; Zhou W. et al., 2016; Dai et al., 2016). A previous study showed that LW could promote AHN in adult rats (Lee et al., 2005). LW-AFC is an active fraction combination derived from LW. Previous studies had revealed that LW-AFC ameliorated cognitive and emotional dysfunctions in model mice of Alzheimer's disease or stress (Zeng et al., 2019a; Huang et al., 2019; Cheng et al., 2020). In the present study, we further investigated the effects of LW-AFC on cognitive and emotional dysfunctions and AHN impairment along the dorsal–ventral axis induced by cranial irradiation. Our results showed that LW-AFC ameliorated behavioural dysfunctions in the contextual fear conditioning and open field tests on the first day after irradiation. In addition, in the tail suspension test, LW-AFC reduced the immobility time in IR mice. LW-AFC also improved cranial irradiation–induced impairment in the proliferation of neural stem cells and reduction in the number of immature neurons preferentially in the dorsal hippocampus on the first day after irradiation. Taken together, our study showed that LW-AFC ameliorated cranial irradiation–induced cognitive and emotional dysfunctions as well as promoted AHN preferentially in the dorsal hippocampus, and the amelioration of behavioural dysfunctions was related to the promotion of AHN.

Apart from the beneficial effect on AHN, we also investigated the effect of LW-AFC on the neurogenic microenvironment in the hippocampus of IR mice. The neurogenic microenvironment is composed of neural stem cells and their surrounding cells in the hippocampus, as well as the regulatory factors secreted by them (Zhao et al., 2008; Ming and Song, 2011; Nicola et al., 2015), and plays an important role in regulating the different stages of AHN (Mosher and Schaffer, 2018; Arredondo et al., 2020). Several reports indicated that regulating the changes in the neurogenic microenvironment might be an effective strategy for promoting AHN and improving cognitive and emotional dysfunctions following cranial irradiation, such as the reduction of inflammation and oxidative stress or the application of cytokines (Monje et al., 2003; Manda et al., 2009; Ramanan et al., 2009; Jenrow et al., 2010; Kim et al., 2010). Our research suggested that cranial irradiation induced disturbance in the neurogenic microenvironment of the hippocampus which was characterized by the increase in the level of oxidative stress and inflammation and the reduction in the level of neurotrophic and growth factors. It showed that LW-AFC increased the contents of SOD, GSH-Px, GSH, and CAT and decreased the content of MDA in the hippocampus of IR mice, as well as increased the contents of BDNF, IGF-1, and IL-4. The results

demonstrated that LW-AFC ameliorated the disturbance in the neurogenic microenvironment in the hippocampus by reducing the level of oxidative stress and increasing the level of neurotrophic and growth factors as well as anti-inflammatory factor. Interestingly, LW-AFC promoted AHN and ameliorated cognitive and emotional dysfunctions despite having no effect on cranial irradiation–induced elevation in hippocampal proinflammatory cytokines, suggesting that the molecular signals triggered by LW-AFC could override this inhibitory effect.

In this study, there are some issues to be illustrated. First, AHN involves proliferation, differentiation, maturation, and migration with neural markers which can be used to quantify different stages (Faigle and Song, 2013). The proliferation and differentiation of neural stem cells have higher vulnerability to cranial irradiation (Bellinzona et al., 1996; Nagai et al., 2000; Lonergan et al., 2002; Andres-Mach et al., 2008). They are the two most important stages of AHN, which are closely linked to the generation of newborn neurons. They are positively correlated on the whole. Upon activation, the population of quiescent neural stem cells gives rise to a proliferative pool of neural stem cells and these cells continue to differentiate into immature neurons that express the marker of DCX in a multistep process (DeCarolis et al., 2013; Braun and Jessberger, 2014; Sibbe and Kulik, 2017). Second, there are currently no clinically available drugs for cranial irradiation treatment by promoting AHN. Although amifostine is the only proved radioprotective drug for radiotherapy in patients, its main pharmacological feature is antioxidant activity and it has many side effects (Anné, 2002; Vacha et al., 2003). We also searched the literature of similar studies and have found no suitable positive drug (Ji et al., 2014; Fan et al., 2017; Ji et al., 2020). Therefore, we also did not design positive drug treatment in our study. In addition, we have detected the neurogenic microenvironment in the whole hippocampus to observe the overall effect of LW-AFC. The change of the neurogenic microenvironment along the dorsal–ventral axis of the hippocampus is not examined. Further study is needed to fully solve this inadequacy.

CONCLUSION

In conclusion, first, our research demonstrated that cranial irradiation impaired AHN in both the dorsal and ventral hippocampus, which significantly contributed to the pathogenesis of cognitive and emotional dysfunctions following cranial irradiation. Second, LW-AFC significantly ameliorated cognitive and emotional dysfunctions after cranial irradiation. The underlying mechanisms included promoting AHN preferentially in the dorsal hippocampus and ameliorating disturbance in the neurogenic microenvironment. LW-AFC might be a promising therapeutic agent to treat cognitive and emotional dysfunctions induced by cranial irradiation (**Figure 9**), having the hope of safeguarding the health of brain tumour patients receiving cranial radiotherapy. Third, LW-AFC displayed a region-specific effect of neurogenic response along the dorsal–ventral axis of the hippocampus. We developed a scientific method to investigate whether TCM and its bioactive components could promote AHN accurately in different subregions of the hippocampus. Finally, the formula of TCM is advantageous in the treatment of complicated diseases for the integrative and synergistic effects exerted by its multiple components through multitargets. Our study provided a scientific method to develop the medication against cognitive and emotional dysfunctions induced by cranial irradiation from the resource of TCM.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institute Animal Care and Use Committee (IACUC) of the National Beijing Center for Drug Safety Evaluation and Research (NBCDSER) (No. 2018-030).

AUTHOR CONTRIBUTIONS

TS, SF, WZ, and YZ were involved in designing the study. MW carried out all experiments, analysed the data, and wrote the article. LZ and CW helped to revise the article. SC helped to do the behavioural testing. TS, WZ, and YZ participated in revising the article and approving the submitted version. All authors had read and agreed to the published version of the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.717719/full#supplementary-material

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