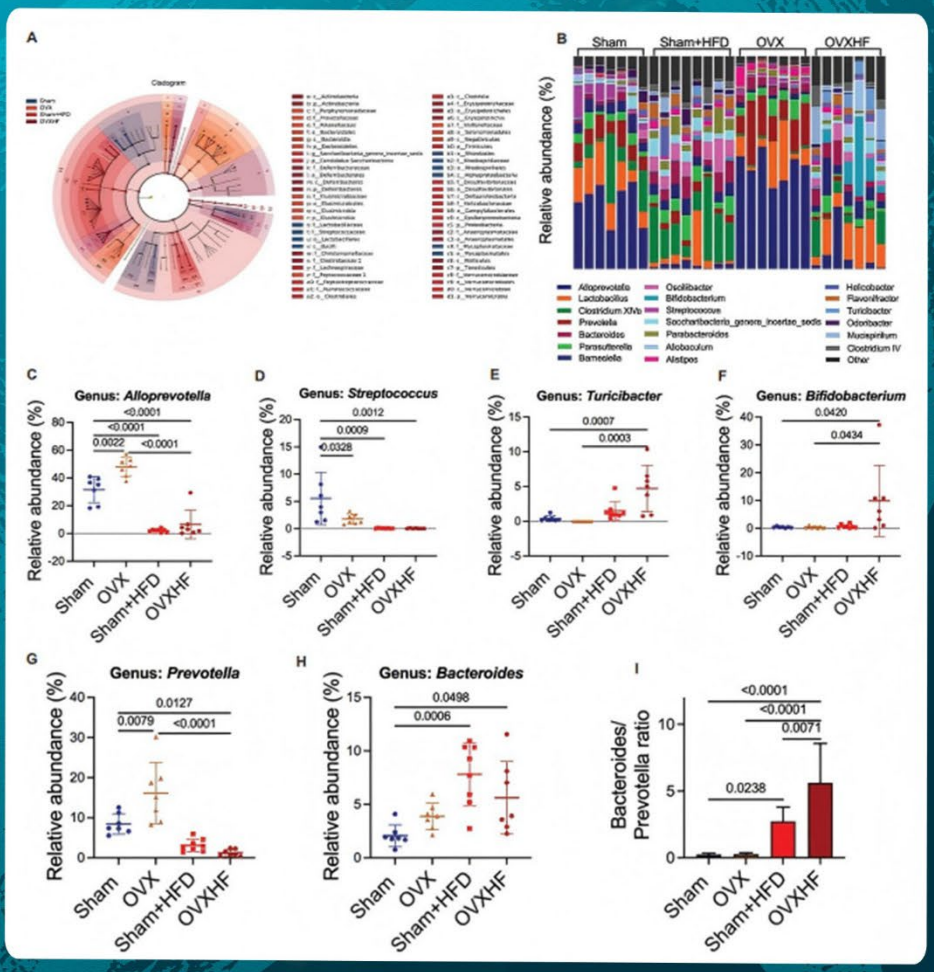
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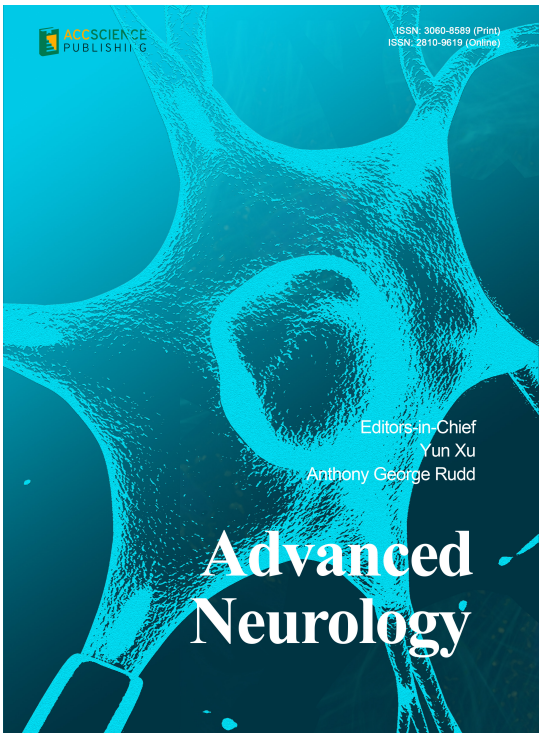
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Editors-in-Chief

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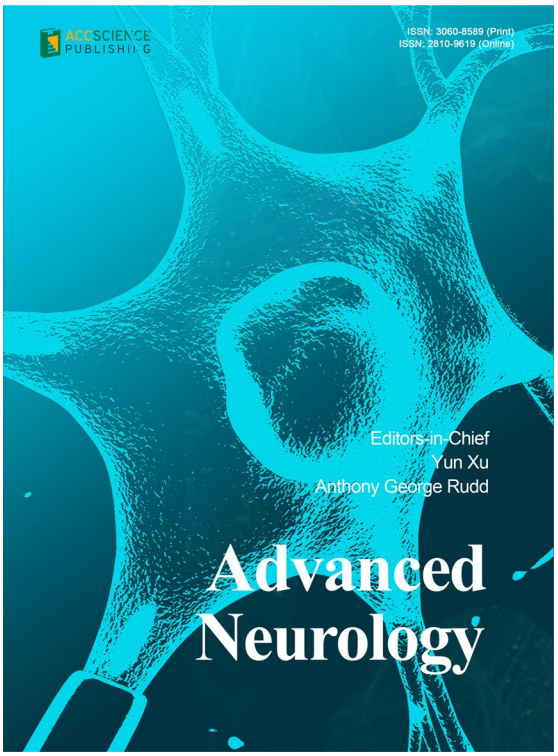
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REVIEW ARTICLE

Effectiveness of non-pharmacological interventions for neurodegenerative disorders: A comprehensive narrative review

Mariam Moselmani[1], Aya Kawssan[2], Fatima Mortada[2], Ahmad Atieh[2], Hayat Chahine[2], Abdallah Alahmad[2], and Hiba Hamdar[3]*

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Abstract

Age-related neurodegenerative disorders encompass a range of diseases in the central nervous system characterized by neuronal death, disrupted neural connections, altered neurotransmitter secretions, and notable features of proteinopathy. The increasing prevalence of age-related neurodegenerative diseases (NDs) highlights the need for effective therapeutic strategies. This review aims to advocate for non-pharmacological interventions (NPIs), such as adopting healthy lifestyle habits, engaging in physical exercises, ensuring sufficient quality sleep, and maintaining cognitive activity, over pharmacological treatments. A literature search was conducted across PubMed, Scopus, Web of Science, and Google Scholar. Studies were included if they focused on NPIs in ND treatment, were published in peer-reviewed journals, and were written in English. Compared to pharmacological options, many of the NPIs have demonstrated satisfactory clinical outcomes in delaying the progression of neurodegenerative disorders or preventing them in certain cases. Specific dietary patterns, such as the Mediterranean-Dietary Approaches to Stop Hypertension Diet Intervention for Neurodegenerative Delay and Mediterranean diet, have been shown to reduce cognitive impairment while mitigating neuroinflammation and oxidative stress. Regular physical exercise promotes an anti-inflammatory state and enhances the secretion of anti-stress hormones like dopamine, thereby fostering neuroplasticity. In addition, obtaining adequate quality sleep and adhering to a consistent circadian rhythm is crucial for memory consolidation and overall cognitive health. Moreover, stress management techniques, such as meditation and yoga, improve cerebral blood flow and help preserve brain structure. Cognitive training and social engagement further contribute to the delay and attenuation of neurodegeneration, thus maintaining cognitive health. Although NPIs show promise in slowing ND progression, further research is needed to validate their effectiveness.

Keywords: Neurodegeneration; Alzheimer; Pharmacological interventions; Non-pharmacological interventions; Memory consolidation; Cognitive function; Neuroinflammation

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1. Introduction

The mortality and disability burden associated with neurological disorders is increasingly recognized as an international public health concern, with projections indicating a substantial rise in prevalence over the coming decades.[1] A review by Erkkinen *et al.*[2] on individual epidemiology of neurodegenerative diseases (NDs) reveals Alzheimer's disease (AD) is the most prevalent, affecting over 24 million worldwide, with its prevalence projected to double every 20 years. Parkinson's disease (PD), the second most common neurodegenerative condition, is experiencing the fastest growth in both incidence and prevalence globally. Less common conditions include amyotrophic lateral sclerosis (ALS), with an annual incidence of 1 – 3 cases/100,000, and Huntington's disease, with a prevalence of 5 – 10/100,000 among populations of European ancestry.[2] Neurodegeneration is recognized as the primary pathophysiological alteration associated with the majority of brain-related disorders.[3] This process is linked to synaptic and neuronal dysfunction, as well as the accumulation of physiochemically altered protein aggregates in the encephalon, playing a crucial role in the pathophysiology of various neurological diseases.[3] Neurodegeneration typically co-occurs with cognitive impairment, intellectual decline, and dementia.[4]

Diseases characterized by neurodegeneration are collectively referred to as NDs. Prominent examples include AD, PD, Huntington's disease, and ALS. While AD and dementia have conventionally been associated with older populations,[3] their prevalence is increasingly observed across all age groups. This trend can be attributed to contemporary lifestyles marked by digital engagement, higher rates of hypertension, widespread hearing impairments, smoking habits, midlife obesity, depression, physical inactivity, and diabetes.[4]

A variety of pharmacological agents are available to treat NDs, including apomorphine, baclofen, donepezil, and entacapone.[2] Although apomorphine is rarely used today, it can be a lifesaver for patients with advanced PD experiencing freezing episodes. Baclofen is used to manage spasticity associated with multiple sclerosis and ALS, but it is not necessary for other NDs.[5] Donepezil has been shown to improve the quality of life for patients at all stages of AD and can delay the need for nursing home placement.[6] Entacapone is a standard treatment for patients with moderate-to-severe PD, improving quality of life and reducing motor fluctuations.[7]

While non-pharmacological interventions (NPIs) cannot replace pharmacological treatment, they can serve as adjunct therapies for managing NDs.[8] Research into NPIs has gained significant traction in recent years within the global healthcare community. Studies indicate that patients with dementia display enhancements in cognitive abilities through non-pharmacological treatment.[9] In 2017, the Collaborative University Platform for Evaluating Health Prevention and Supportive Care Programs defined NPIs as evidence-based, non-invasive methods aimed at improving human health, focusing on prevention, management, or remedying medical issues. These interventions encompass a wide range of items, practices, initiatives, or offerings that are familiar to users.[10] NPIs include lifestyle modifications such as physical activity and nutrition, as well as cognitive stimulation techniques such as mental training and deep brain stimulation.[11]

Some research suggests that dietary supplementation can enhance cognitive performance in AD patients. Several studies have shown that diets rich in antioxidants and anti-inflammatory components, such as fruits, nuts, vegetables, and seafood, may help prevent age-related cognitive decline and reduce the risk of developing neurodegenerative disorders.[11] Epidemiological data and clinical and pre-clinical research highlight omega-3 polyunsaturated fatty acids (n-3 PUFAs) as one of the most promising dietary approaches for managing NDs.[12] Increased consumption of total n-3 PUFAs, particularly docosahexaenoic acid (DHA) from seafood, has been associated with a significant reduction in the incidence of AD and dementia. Furthermore, multiple studies have shown that higher folate or Vitamin B6 consumption reduces the risk of developing AD.[7] The most critical biomarkers for AD include amyloid precursor protein, apolipoprotein E4, tau protein, and amyloid-beta (Aβ), all of which are linked to dietary factors and physiological processes.[13]

Physical activity demonstrates significant potential in modulating key pathological mechanisms of AS. A substantial body of preclinical research demonstrates that exercise enhances Aβ clearance, decreases its production, augments tau degradation, and suppresses tau phosphorylation – processes central to AD's neuropathology.[14] In addition, extensive research has shown that physical activity increases brain-derived neurotrophic factor (BDNF) and maintains brain size, especially in the hippocampus. A systematic review determined that aerobic exercise preserves hippocampal volume, whereas a number of the included papers indicated it may even induce structural expansion in older adults.[15] Social interaction emerges as another NPI, with research suggesting that it reduces cognitive impairment rates.[16] A study revealed that older individuals who participate in frequent social activities or have vast social connections have better cognitive performance compared to those who do not.[17]

Notably, sleep deprivation contributes to Aβ buildup in the human brain. A study by Shokri-Kojori *et al.*[18] found that following sleep deprivation, Aβ levels increased in the hippocampus, a brain area highly sensitive to AD neuropathology, as well as in the thalamus, which shows elevated Aβ levels in the early stages of AD.[18]

As research highlights the limitations of non-pharmacological treatments, they still offer a promising avenue for improving care and quality of life for individuals with NDs. Hence, the objective of this study is to study the role of non-pharmacological therapies in the treatment of neurodegenerative illnesses and compare their efficacy to pharmaceutical interventions. This review aims to provide a comprehensive understanding of how lifestyle changes, cognitive stimulation, and early detection strategies can supplement or potentially outperform traditional drug therapies in managing and treating neurodegenerative conditions.

2. NPIs for NDs

2.1. Overview of ND progression

Neurodegenerative disorders are severe, mostly incurable conditions that are frequently associated with aging.[19] Existing therapies have limited efficacy and focus on alleviating symptoms rather than dealing with the underlying cause or progression.[20] The three most prevalent disorders, AD, PD, and ALS, exhibit distinct pathological hallmarks and clinical trajectories.[21]

Research indicates that the buildup of Aβ and tau proteins plays a key role in AD development.[22,23] AD is characterized by the production of Aβ containing plaques in the brain, which are related to neurofibrillary tangles composed of hyperphosphorylated tau proteins.[23-25] Plaque formation alters hippocampal circuitry, impairing short-term memory consolidation into long-term traces.[26] AD causes widespread neuronal loss, incorrect synaptic connections, and impairment of the critical neurotransmitter systems required for brain activities, such as memory. Thus, selective memory impairment is the most prevalent clinical indication of AD in its early stages. Furthermore, hippocampal and medial temporal lobe-dependent processes, such as declarative episodic memory, are frequently disrupted. Other early clinical manifestations include impaired executive function, judgment, and problem-solving.[27]

PD is a progressive neurological disorder that leads to tremors, muscle stiffness, unsteady walking, and difficulties in balance and coordination.[28] It is characterized by the presence of neuronal aggregates known as Lewy bodies and Lewy neurites, alongside significant cell loss in the substantia nigra and other brain regions. PD is classed as a synucleinopathy as Lewy bodies are primarily composed of aggregated and mis-folded α-synuclein.[29] On the other hand, ALS, also known as motor neuron disease or Lou Gehrig's disease, is a degenerative condition affecting nerve cells in the brain and spinal cord. ALS leads to muscular weakening and paralysis.[30,31] as motor neurons degrade progressively before eventually dying.[32] This loss of motor neurons disrupts the transmission of signals from the brain to muscles. Genetic research has identified over 30 genes linked to ALS, with mutations in four major genes (*C9orf72*, *TARDBP*, *SOD1*, and *FUS*) accounting for more than 70% of cases.[32] These genes encode proteins involved in critical motor function roles, including DNA repair, homeostasis, mitochondrial activity, and glial cell function. Dysfunction in these processes leads to the degeneration of motor neurons in ALS.[3]

Neurodegenerative disorders exhibit varying intensity based on the severity and stage of the illness, with some conditions posing life-threatening risks. As the brain regulates all bodily functions, NDs can affect fundamental and complex human activities, such as speech, mobility, coordination, equilibrium, urinary functions, and cognition. Treatments for NDs typically aim to improve symptoms, relieve pain, and restore functional activities such as walking.[32]

The approach of treatment is often disease-specific, with several techniques currently approved to either address the pathophysiology of the disease or improve the symptom's impact on patients' quality of life.[33] Pharmacological treatments are generally considered first-line therapies; however, they face challenges in effectively reaching their target sites within the brain.[34] Current therapies for NDS primarily focus on reducing the disease's progression rather than modifying the root causes. Moreover, the role of the blood-brain barrier (BBB) in drug absorption in patients with NDs remains a topic of debate, as some studies suggest that the sophisticated morphology of the BBB, combined with the limited efficacy of most drugs, restricts therapy options for NDs.[35] However, this theory is not entirely accurate, and ongoing research continues to explore the potential impact of the BBB on therapeutic outcomes.

In light of these challenges, there is a growing need to integrate non-pharmacological strategies into treatment plans. NPIs aim to enhance patients' responsiveness, quality of life, and ability to perform daily activities.[36] These interventions often include functional rehabilitation, aerobic physical exercise, cognitive training (CT), occupational therapy, and changes in lifestyle routines, such as nutrition regimen and quality of sleep. Brain

stimulation is another strategy that will be discussed further.[37] Considering this, there is a compelling need to understand the processes of non-pharmacological treatment options in managing NDs.

2.2. Nutrition

Diet type, in conjunction with various factors, may impact neural development and cognitive performance. Numerous studies have established a link between a nutritious diet and enhanced cognitive abilities.[38] Nutrients are fundamental components for cell proliferation, DNA synthesis, neurotransmitter and hormone metabolism and are essential for enzyme systems in the brain.[39] Research has provided substantial evidence that adherence to dietary patterns such as the Mediterranean diet, Dietary Approaches to Stop Hypertension (DASH), Mediterranean-DASH Diet Intervention for Neurodegenerative Delay (MIND), and Modified Mediterranean Ketogenic Diet can lower the risk of developing AD and related dementia, while also hindering disease progression. These diets are particularly beneficial for cognitive health as they contain polyunsaturated fat, increased fiber, various minerals, and compounds with neuroprotective effects. Several studies have found that particular nutrients in these diets have preventative or therapeutic benefits for AD.[40]

The MIND diet is gaining popularity for its neuroprotective benefits,[41] characterized by a healthy plant-based dietary pattern that includes ten brain-healthy foods (green leafy vegetables, other vegetables, berries, fresh fish, poultry beans, whole grains, nuts, wine, and olive oil) and five limiting foods high in animal and saturated fat (butter and margarine, cheese, red meats, pastries and sweets, and fast fried food).[42] Since its introduction in 2015, longitudinal studies have demonstrated that the MIND diet has protective effects on cognitive impairment,[43] dementia,[44] and even brain structure.[45] In animal experiments and laboratory research, the MIND diet blocks foods high in saturated and trans-fat, which can lead to BBB dysfunction, increased cerebrovascular inflammation, Aβ accumulation, and impaired learning capacity and memory.[46]

It is apparent that a typical Mediterranean diet rich in extra virgin olive oil (EVOO) and nuts, which contain several health-promoting components, not only reduces neuroinflammation and oxidative stress but also enhances immunological function. The antioxidant properties of olive oil are attributed to compounds such as tyrosol, oleuropein, and hydroxytyrosol, which exhibit neuroprotective, antioxidant, and anti-inflammatory effects.[44] In addition, oleic acid can be converted to nitro-oleic acid, which reduces neuroinflammation and oxidative stress by inhibiting nuclear factor kappa-B (NF-κB) activation and stimulating the nuclear factor erythroid 2-related factor 2 (Nrf2) transcriptional factor. Moreover, neuroinflammation in neurological diseases can induce pain by activating primitive nociceptive and somatosensory neurons.[47] EVOO molecules have been proven to activate transient receptor potential ankyrin 1, reducing pain intensity by activating nociceptive neurons via these receptors. Nitro-oleic acid, an endogenous peroxisome proliferator-activated receptor gamma ligand, has vascular protective effects and can downregulate the expression of NF-κB proinflammatory genes as well as upregulate Nrf2, an antioxidant transcription factor.[48]

Polyphenols found in these diets have been shown to improve cognitive health through various mechanisms. They can increase cerebral blood flow, lower oxidative stress, reduce neuroinflammation, boost neurogenesis, and enhance neuroplasticity.[47] Whole grains boost cognitive function through their cardiometabolic and gastrointestinal impacts, as indicated by the gut microbiota-brain axis.[49] In addition, n-3 PUFAs, especially those obtained from aquatic sources, as well as mono- and polyunsaturated fatty acids found in nuts, provide protective benefits through anti-inflammatory properties, reduced blood pressure, and improvements in endothelial functions.[50]

Vitamins, including B9, B12, and E, have been implicated in neurogenesis in adults due to their antioxidant properties.[51] Vitamin B12 is an essential element for healthy neurological function as it aids in homocysteine regeneration, methionine synthesis, methylation process, and serotonin production.[3] Low Vitamin B12 levels can impair homocysteine regeneration to methionine and methyl malonyl-CoA (MMA) isomerization to succinyl-CoA, resulting in elevated circulating levels of homocysteine and MMA.[52] A rise in homocysteine can also enhance the amount of reactive oxygen, leading to neuronal apoptosis and depressive symptoms.[53] Furthermore, elevated levels of iron in the brain have been linked to cognitive decline in older adults and those with AD[54] due to its association with dopaminergic neurotransmission and dopamine production.[55] The buildup of iron may cause neurotoxicity and synaptic toxicity through oxidative stress and ferroptosis, resulting in hyperactive dopamine neurotransmission.[21]

Research suggests that taking Vitamin D supplements and getting at least 15 min of sunshine every week can help prevent PD.[56] A review found that high Vitamin D levels can enhance balance and postural stability and reduce fall risks in young PD patients but not in elderly people.[57] Besides, high serum Vitamin D levels have been linked to improved cognition in PD individuals without dementia.[51] Vitamin

D therapy can decrease dopaminergic neurodegeneration and microglia-mediated neuroinflammation by decreasing the expression of inducible nitric oxide synthase and toll-like receptor-4 while increasing the mRNA levels of anti-inflammatory cytokines (interleukin [IL]-10, IL-4, and transforming growth factor-beta), as well as markers such as CD163, CD206, and CD204.[58] In addition, Vitamin D has been shown to reduce oxidative stress and inflammation, which can benefit AD. Specifically, 1,25(OH)2D3, the active form of Vitamin D, has demonstrated anti-inflammatory effects in brain pericytes by modulating transcriptional processes, suggesting that Vitamin D may directly protect brain capillaries in the presence of persistent inflammation, such as that observed in AD.[59]

Furthermore, the consumption of unhealthy foods and beverages, such as highly processed meat, refined grains, refined sugars, and snacks high in modified sugar, should be addressed in recommended dietary plans, as these items have been strongly linked to an elevated risk of cognitive damage, AD, and related dementias.[60]

On the other hand, caloric restriction has shown promising effects in reducing age-related behavioral deficits, including brain aging, and should be considered for further investigation.[61]

2.3. Exercise

Physical exercise (PE) has been recommended as one of the most effective lifestyle remedies for both normal aging individuals and patients with NDs, such as AD and PD.[62] Clinical studies have shown that physical exercise is an effective treatment for AD patients, and it may serve as a disease-modifying therapeutic method.[63]

Previous research revealed that exercise improved many symptoms in individuals with PD, including executive dysfunction, psychological issues, and cognitive decline. The dose-response of treadmill exercise to PD showed that only high-intensity exercise effectively reduced motor symptoms in PD patients.[64]

Physical exercise provides numerous positive benefits, including promoting neurotrophic factor synthesis, exhibiting anti-inflammatory effects, and inducing angiogenesis. Research suggests that exercise can reduce Aβ deposition and improve tau pathology in the brain.[65]

Physical exercise reduces chronic oxidative stress while stimulating mitochondria biogenesis and enhancing autophagy in PD. In addition, it can stimulate the production of neurotransmitters such as dopamine and trophic factors such as glial-derived neurotrophic factor, insulin-like growth factor-1, BDNF, and fibroblast growth factor 2.[66] By modulating neurophysiological pathways, PE contributes to autophagy, neuronal plasticity, neurogenesis, and anti-oxidant defense systems. It also reduces neuronal apoptosis and neurodegeneration.[67] Regular PE may also cause neuroplastic alterations in the human brain, although the effects vary greatly between individuals.[68] In addition, regular PE stimulates autophagy and enhances neurological function.[66]

Many experimental experiments using homogenized tissues to measure the levels of various neurotransmitters have shown that changes in monoamine production and metabolism occur during exercise.[69] As stated by Lin and Kuo,[70] the three primary monoamine neurotransmitters regulated by exercise are dopamine, noradrenaline, and serotonin (hydroxytryptamine or 5-HT),[70] and their release increases during physical activity.

Literature mainly discusses four types of exercises: (i) aerobic exercise (e.g., running and walking), (ii) resistance exercises (e.g., weightlifting and the use of external weights or body weight), (iii) balance exercises (e.g., Tai Chi), and (iv) flexibility exercises (e.g., yoga). While most research focused on aerobic exercise, all four types of training have shown an impact in reducing depression and enhancing cognitive function when performed with sufficient intensity.[71] Silverman and Deuster[72] identified that regular physical activity impacts several biological pathways: (i) enhancing neuroendocrine and physiological responses to psychosocial and physical stressors, (ii) acting as a buffer against stress and stress-related diseases/chronic diseases, (iii) promoting an anti-inflammatory state, and (iv) enhancing neuroplasticity and growth factor expression.[72]

Aerobic exercise significantly raises adenosine triphosphate-binding cassette transporter 1 mRNA expression, which may improve cognitive performance and mitigate AD symptoms.[73] Collectively, these findings suggest potential treatments for age-related neurodegenerative illnesses such as AD.[66]

BDNF is a neurotrophic factor that promotes differentiation,[74] maturation,[75] and survival of neurons in the nervous system.[76] It also exerts neuroprotective effects under adverse conditions such as glutamatergic stimulation, cerebral ischemia, hypoglycemia, and neurotoxicity.[77] Serum/plasma BDNF levels have been shown to increase following both aerobic (dance and sports) and anaerobic (Pilates) activities.[78] Håkansson *et al.*[78] conducted a study in which healthy volunteers participated in moderate-intensity aerobic activity. BDNF levels in the circulation of elderly participants increased following 35 sessions of aerobic exercise, as measured by enzyme-linked immunosorbent assay.[78] BDNF generated peripherally can function as a metabolite in response to skeletal muscle activation.[79] This molecule is transported

by the bloodstream across the BBB, where it is activated in the central nervous system.[79] PE affects a variety of physiological features, including hippocampus insulin signaling, autophagy, antioxidant and anti-inflammatory responses, and longevity of cells and death paths.[66]

2.4. Sleep quality

Sleep is a crucial physiological mechanism for supporting general health and well-being.[80] It is a period for healing processes, memory consolidation, and the regulation of various biological systems. However, individuals with NDs often suffer from altered sleep patterns, including insomnia, excessive daytime drowsiness, and disordered sleep architecture.[81] These sleep disruptions not only influence the quality of life of patients and caregivers but may also contribute to the progression and severity of underlying NDs.[82]

The relationship between AD and sleep issues is bidirectional. AD leads to significant sleep deprivation and interruptions due to the degeneration of the suprachiasmatic and cholinergic nuclei.[83] These anomalies may affect cognitive function by reducing the brain's ability to remove Aβ.[81] NDs associated with abnormal tau accumulation, such as AD, are usually followed by sleep disorders. These disorders often arise from damage to brain regions that regulate sleep, as well as from other psychiatric, medical, and primary sleep disturbances, medication-related effects, and insufficient light exposure and activity during the day.[84]

Cognitive-behavioral therapy (CBT), sleep hygiene routines, and bright light therapy (BLT) are frequently used to address sleep issues. Among non-pharmacological treatments for chronic insomnia, CBT shows the greatest promise. The long-term benefits of this approach stem from enhancing the patient's ability to support and promote the body's natural sleep processes.[85]

Abnormalities of the circadian rhythm, such as delayed sleep phase syndrome, which results in a change in the normal sleeping cycle beyond socially acceptable limits, are treated with BLT.[86] It has been suggested that BLT may work by either raising synaptic serotonin – potentially sourced from the serotonin-rich midbrain connected to retinofugal pathways – or by resolving the winter circadian rhythm phase delays.[87]

2.5. Stress regulation

Stress exacerbates disease development and increases symptoms in NDs such as AD, PD, and Huntington's disease. Various methods for alleviating stress (such as yoga and meditation) have protective properties against neurodegeneration.[88] Anti-stress therapies, such as natural medicines, exercises, yoga, and meditation, are becoming increasingly important for restoring health and vitality. In terms of neurodegeneration, reducing stress during early life may assist in lowering the risk of developing dementia later in life. Thus, decreased stress may be employed as a preventative measure. Interventions aimed at reducing stress have been employed in individuals with various NDs.[88] Meditation has been found to improve localized cerebral blood flow in the frontal and anterior cingulate areas of the brain while promoting performance in the brain's executive attentional pathway.[89]

Cognitive behavioral therapy is a collaborative psychological method that targets negative automatic thoughts and enhances awareness or beliefs. It effectively engages patients' cognition and actions.[90] CBT programs have favorable effects in reducing both anxiety and depression symptoms among PD patients compared to those not receiving CBT. A previous systematic review suggests that CBT holds potential clinically relevant benefits and provides additional evidence for its efficacy in treating depression and anxiety in chronic neurological conditions, including PD.[91] Recent studies indicate that yoga can positively influence overall brain health by reducing stress levels in healthy older adults, older individuals with mild cognitive dysfunction, and patients with dementia. Yoga practice has been shown to improve cognitive function, affective interaction, and physical abilities among healthy elderly populations while positively impacting total brain volume, neocortical gray matter thickness, and functional connectivity between different brain areas in subjects with mild cognitive dysfunction.[92]

3. The role of cognitive stimulation

3.1. CT

In the treatment of NDs, such as PD and AD, cognitive stimulation is a significant NPI. The many facets of cognitive stimulation are explored in this section, along with its impact on NDs.

CT and interventions have consistently been shown to improve cognitive abilities.[93] Sensorimotor, cognitive, and physical performance deteriorate with age, but improvements can be achieved with training and exercise, suggesting that age-related changes are modifiable.[94] This training involves activities that require extensive neuronal activity and regulation, such as dancing, language learning, and music training.

Dancing is increasingly recognized as a potentially neuroplasticity-inducing intervention due to its multifaceted nature. Moderate physical activity can prevent age-related decline when combined with cognitive, social,

and emotional engagement.[94] A study elucidated that a 6-month dance intervention can significantly improve several aspects of physical and cognitive functioning.[94] Language learning engages different cognitive processes, including memory and problem-solving, thereby enhancing cognitive reserve and versatility. Studies have shown that bilingualism may delay the onset of dementia by about 4 years compared to monolingual individuals. However, this protective effect does not affect the rate of cognitive decline once dementia has begun.[95] In addition, music has also been shown to attenuate the progression of NDs. A study showed that music-based training once per week over 6 months was associated with improved cognitive functions and decreased anxiety in community-dwelling older adults compared to those not receiving training.[96]

3.2. CT activities

3.2.1. Dancing

Dancing is a physical activity that engages multiple brain regions, including the cerebellum, basal ganglia, and motor cortex, due to its demand for complex coordination, balance, and rhythm. Studies have proved the potential of dancing to enhance neuroplasticity, which can lead to improvements in executive functions, attention, and memory, thereby delaying the onset of dementia.[94]

The intricate systems involved in executing dance movements influence several neurotransmitter systems. Dopamine, a neurotransmitter crucial for movement and reward pathways in the brain and a key factor in many NDs like PD, is greatly influenced by dancing. Studies have shown that dopamine release influenced by dancing can improve motor function and reduce symptoms such as bradykinesia and rigidity in patients diagnosed with PD.[97]

Serotonin is another neurotransmitter released during physical activities like dancing and is essential in enhancing mood and attenuating the symptoms of depression and anxiety, which are common in NDs.[98] In addition, endorphins released by dancing promote a sense of well-being and offer pain relief for these patients.[99]

Pharmacological interventions for NDs, such as PD, often involve levodopa and several other dopamine agonists. While these medications are effective in managing motor symptoms, they may cause side effects such as dyskinesia and psychiatric problems.[100] In contrast, dancing offers a holistic approach with much fewer side effects, thus enhancing the quality of life through physical and emotional benefits.

3.2.2. Language learning

Learning a new language requires the engagement of multiple brain regions, such as the hippocampus, temporal lobe, and frontal lobe, making it a cognitively demanding task. Language learning has been shown to increase the integrity of central white matter tracts and the density of gray matter, which are essential for neuronal transmission.[101]

Acetylcholine, which is an abundant neurotransmitter, is critical for synaptic plasticity and plays a key role in memory formation called "long-term potentiation." Language learning has been shown to enhance cholinergic function, thereby improving memory and cognitive functions.[102] Dopamine is also a crucial neurotransmitter and the source of several NDs. The challenge and reward system offered while learning a language stimulates the release of dopamine, thus reinforcing learning and motivation.[103] Language learning promotes the glutamatergic activity conducted by glutamate, which is essential in synaptic activity and plays a central role in the long-term potentiation and enhancement of cognitive function.[104]

Medications from the class cholinesterase inhibitors are commonly used to increase acetylcholine levels and improve cognitive function in AD patients. While these drugs can be effective in the early stages of the disease, they can cause gastrointestinal side effects and have very limited efficacy in the advanced stages of the disease.[105] In contrast, language learning offers a central non-invasive enhancement of cholinergic activity with broad benefits and no therapeutic side effects.

3.2.3. Music

There are several ways a patient can engage with music: listening, instrument playing, and singing. Music therapy has consistently shown its ability to improve cognitive functions, alleviate anxiety, and promote well-being in patients with NDs.[106]

Engagement with music elevates several neurotransmitters. For instance, music can increase oxytocin levels, especially in group settings, which is important for stiffening emotional connections and promoting social bonding.[107] Dopamine levels are greatly increased when listening to and playing music, and given the importance of dopamine, music plays an important role in mood enhancement, cognitive improvement, and reward processing.[108] Moreover, music therapy can reduce stress and improve mood through the manipulation of serotonin and endorphin levels.[109] While many medications are prescribed to patients with NDs to manage mood and anxiety symptoms, such as antidepressants and anxiolytics, these medications are associated with significant side effects. In addition, they may not effectively address the social and emotional aspects of care. In contrast, music

therapy offers a holistic approach that engages multiple neurotransmitter systems with far fewer side effects.[109]

3.2.4. Social interactions

Social interactions play a crucial role in cognitive health. The trend of cognitive decline has been shown to be attenuated by engaging in social activities, attending community events, and maintaining marital status. A study indicated that people living alone or having no close social ties have a 1.5-fold increased risk of dementia. Moreover, compared to married people, people living a single life have a 1.9-fold increased risk of dementia.[110] Highlighting the importance of marital status, a previous study reveals that people cohabiting with a partner in mid-life are less likely than all other categories (single, separated, or widowed) to show cognitive impairment later in life, particularly between ages 65 and 79.[111] Marital status and close relationships greatly impact cognitive functions and mental health. Studies have shown that married individuals have a lower risk of developing NDs, as emotional support helps reduce stress and contributes to cognitive health and well-being.[112]

Oxytocin, which is the "love hormone," is central to social interactions, which is why close relationships can increase oxytocin levels. Oxytocin can suppress cortisol secretion systematically, thus attenuating stress and its detrimental effects on the brain.[113] Dopamine and serotonin levels are also elevated during close social interactions, which aid in improving mood and cognitive resilience.[114]

Social activity is essential for the maintenance of neural networks, thereby improving cognitive function and emotional health.[115] Engaging in social activities helps increase dopamine levels, providing opportunities for positive reinforcement and motivation, which helps sustain cognitive function.[116] Serotonin, another neurotransmitter stimulated by these activities, plays a critical role in maintaining a healthy mood, especially in patients with NDs, lowering the risk of anxiety and depression.[114] Endorphins also mitigate pain and stress, contributing to well-being and overall health.[117]

Pharmacological treatments for NDs focus on the pathological aspects of the disease and do not adequately address the psychological aspects of these conditions. Social support is essential as it increases oxytocin, dopamine, and serotonin and provides a protective effect against cognitive decline (Table 1).[118] Pharmacological treatments, therefore, fail to offer the same holistic benefits provided by social interactions.

3.2.5. Non-invasive brain stimulation

Recently, non-invasive brain stimulation (NIBS) techniques have been applied to healthy and older adults with NDs to improve physiological and pathological cognitive impairments.[119] There are two main forms of this stimulation technique: repetitive transcranial magnetic stimulation (rTMS), which delivers magnetic pulses through the scalp, and transcranial direct current stimulation (tDCS), which applies weak electrical currents to the scalp.[120] Both techniques can either increase or decrease cortical activity depending on the procedure, but for cognitive stimulation, excitatory stimulation is typically used.[119] A meta-analysis by Hsu *et al.*[119] showed that NIBS improved cognitive function in physiological and pathological aging.[119]

In addition, it has been demonstrated that tDCS improves working memory and attention in PD patients.[121] CT, in conjunction with NIBS, has demonstrated the potential to improve cognitive abilities in patients suffering from NDs. A meta-analysis revealed that the combination of NIBS and CT significantly enhanced global cognition in patients with mild cognitive impairment and AD.[122] Similarly, CT paired with tDCS improved working memory and attention in PD patients, with effects persisting at a 12-week follow-up.[123] These cognitive benefits are attributed to NIBS mechanisms such as modulating neuroplasticity, enhancing synaptic efficacy, and influencing neural network connectivity. However, the efficacy of NIBS can vary based on factors such as stimulation parameters, target regions, disease stage, and individual differences. While NIBS techniques are generally well-tolerated, further research is required to optimize protocols for consistent cognitive improvements, which requires further research.

In summary, NIBS methods, such as tDCS and rTMS, show promise in improving cognitive function in neurodegenerative illnesses. Ongoing research aims to improve these strategies and integrate them into treatment plans for illnesses such as PD and AD.

4. Importance of early detection and screening

The risk of dementia is increasing worldwide, with rapid growth in the aging population in Europe, projected to rise to 23.1% by 2030, according to the United Nations.[124] By 2050, up to 16 million older adults are expected to have dementia, associated with estimated costs exceeding 1$ trillion USD.[125] Early identification of NDs, such as AD, PD, and other forms of dementia, is crucial for the effectiveness of both pharmacological and non-pharmacological treatments. Early diagnosis can slow disease progression, improve quality of life, and extend functional independence.[126] Pathologically, AD is characterized by a prolonged preclinical phase without clinical symptoms, an early clinical phase with mild clinical impairment (MCI), and, eventually, a dementia

Table 1. Comparison of pharmacological and non-pharmacological interventions for neurodegenerative diseases

Aspect	Pharmacological interventions	Non-pharmacological interventions
Treatment examples	Levodopa, donepezil, riluzole, and memantine	Exercise, diet, cognitive training, sleep quality, and stress management
Mechanism of action	Targets neurotransmitter levels and reduces symptoms; however, it does not cure the disease	Promotes neuroplasticity, reduces inflammation, and supports cognitive function
Effectiveness	Temporary symptom relief and slows progression but does not stop neurodegeneration	Can delay onset, improve quality of life, and provide long-term benefits
Side effects	Nausea, dizziness, gastrointestinal issues, hallucinations, and liver damage	May include temporary fatigue or soreness from exercise
Long-term benefits	Limited and effectiveness decreases over time	Sustainable improvements in cognitive and physical function
Accessibility	Expensive, requires a prescription, and may not be available in all regions	Widely accessible and can be self-implemented with guidance
Limitations	Does not address root causes and has potential for adverse effects	Requires consistency and lifestyle changes

phase. Within the early stage, cognitive screening methods can be used to test memory, language, orientation, executive function, attention, and visuospatial abilities. These tools are inexpensive, rapid, and non-invasive, making them valuable for identifying adults at risk of AD.

Mini-mental state examination (MMSE) is a widely used screening technique that is effective in identifying cognitive impairment and tracking changes over time. However, it is limited in differentiating between MCI and healthy controls. Other techniques, such as the scenery picture memory test and the memory impairment screen, primarily measure episodic memory, reflecting the diversity of methods used across populations.[124] Neuroimaging has become a critical tool for detecting NDs before the onset of symptoms. Techniques such as magnetic resonance imaging (MRI) and positron emission tomography (PET) have been shown to be capable of differentiating between the common symptoms of ND and psychiatric diseases. This includes the cognitive decline in AD, mood disturbance, and motor function change in PD, which share features such as anxiety, depression, and psychosis, giving evidence for physicians for accurate diagnosis. MRI can visualize smaller brain regions, detect microbleeds with a minimum diameter of 2.5 mm,[126] and quantitatively assess changes in volume and functional connectivity.[127] However, MRI is limited by its lower sensitivity for detecting molecular alteration in the initial phase of ND.[128] PET is capable of detecting tau protein accumulation, a key early indicator of AD due to the accumulation of tau proteins.[129] However, its high cost restricts widespread clinical use.[130] Single photon emission computed tomography accurately identifies PD by visualizing dopamine transporters.[126] The integration of neuroimaging with artificial intelligence is expected to enhance diagnostic accuracy and improve treatment outcomes for NDs.[131] Pharmacologically, interventions such as donepezil, huperzine A, and Vitamin E are used to reduce cognitive decline.[125] Lecanemab, a therapeutic humanized monoclonal IgG1 antibody targeting Aβ plaques. These plaques are early biomarkers in AD, and lecanemab has shown effectiveness in early stages by reducing Aβ plaque aggregation in AD patients.[132]

4.1. Common pharmacological interventions and comparative analysis

Levodopa is a commonly used drug for the treatment of PD and is generally considered better than dopamine agonists or monoamine oxidase inhibitors.[133] However, when compared to apomorphine, a broad-spectrum dopamine agonist without opioid receptor affinity, both demonstrate comparable effects on PD patients. Apomorphine holds a slight advantage in onset duration and incidence of dosage failure (46% with levodopa versus 7% with apomorphine). Subcutaneous apomorphine, with 100% bioavailability, proves more effective than oral administration (bioavailability <4%) and is safer than intravenous induction, which carries a risk of crystallizing.[134] Apomorphine significantly reduced "off times" (36% to 68%) and improved motor rates (19 – 74%) in PD patients.[135] For AD interventions, donepezil (an acetylcholinesterase inhibitor [AChEI]) shows safety and efficacy similar to other AChEIs, like memantine, with significant MMSE improvements after 16 weeks of donepezil treatment. However, global functional gains remain limited.[136] In ALS, riluzole significantly prolongs survival over long treatment periods for ALS patients. Both the medication and the placebo showed similar chances of patients losing more than 50% of their vital capacity, requiring mechanical ventilation, undergoing tracheostomy, or dying. In addition, the treatment showed no improvement in muscle strength.[137]

In addition to their limited effectiveness, pharmacological drugs come with many significant risks. Levodopa, although

a cost-effective medication, has been shown to induce oxidative stress and motor complications.[133] Apomorphine, which is expensive,[138] showed numerous adverse effects (AEs), including nausea (11 – 100%), injection site bruising (13 – 100%), hypotension (2 – 31%), and visual hallucinations (4 – 8%), in addition to some severe AEs like syncope (2%), vomiting (2%), and transient chest pain (15%), which caused some patients to discontinue the treatment.[135] It also showed negative interactions with certain drugs, causing severe hypotension and syncope when given with 5-HT3 agonists and increasing the risk for ventricular tachyarrhythmia with QT/QTc interval (intervals in the electrocardiogram) prolongation drugs.[134] Medications such as fluoxetine and venlafaxine showed significant effects on mood in depressed PD patients; however, they caused AEs such as nausea or hypertension.[139] For the treatment of AD, donepezil has been considered relatively safe, with mainly mild AEs (dizziness, agitation, confusion, diarrhea, and falls), with none leading to death.[140] In a different trial that lasted 72 months, nine out of 273 patients stopped receiving the medication due to gastrointestinal problems.[136] Riluzole for ALS is costly, making it only accessible to the wealthy.[141] The treatment also showed various AEs (nausea, asthenia, elevated liver functions, and low hemoglobin levels). In addition, it induced inflammatory effects on the lungs, liver, and pancreas, all of which improved when the treatment was terminated.[137]

Given the low effectiveness and high risks of pharmacological interventions when treating NDs, non-pharmacological treatments emerged as potentially better and safer treatment options for patients. Exercise has been proven to protect against AD (Table 2). It has been shown to cause cognitive improvements, an increase in Aβ concentrations, and a slower progression of AD. Physical activity also showed improvement in both cognitive and physical capacities in patients with PD.[142] Although physical activity strengthens cardiac health and immune response, heavy exercise is considered a risk factor for respiratory tract infections[143] in addition to other AEs, including falls, hypotension, joint pain, muscle soreness, and fatigue.[144] A ketogenic diet improves AD symptoms by reducing amyloid plaque deposition, improving cognitive functions, and preventing disorders in oxidative phosphorylation. It also showed a neuroprotective influence against PD by decreasing dopaminergic neurodegeneration and mitochondrial deficits in patients. However, some patients had periodical exacerbations of tremors and less frequently showed nausea, dysphagia, body mass loss, headaches, and nephrolithiasis.[145] A Mediterranean diet (fruits, vegetables, and legumes) decreased the risk of AD and PD, while a diet rich in DHA and omega-3 has shown anti-depressive effects and cognitive function improvements.[140] However, some dietary components (especially neutral amino acids) competed with levodopa for absorption by the brain and intestines.[146] Among NIBS, rTMS showed improvement in motor functions, mood, and gait but did not improve cognitive function.[147] The adverse effects of NIBS included fear, headache, tinnitus, dizziness, hearing loss, and fainting.[148]

Proposing a multimodal plan provided better outcomes for patients with NDs. While pairing PE with the wrong diet led to negative effects, combining strenuous exercise with a ketogenic diet showed improvements in cognitive function.[140] In another trial, a combined physical therapy and tDCS approach significantly improved cognitive and executive functions compared to when either was performed alone.[139]

5. Limitation

While NPIs play a valuable role in managing NDs, they come with certain limitations. One of the main challenges

Table 2. Summary of non-pharmacological interventions and their effects on neurodegenerative diseases

Intervention	Benefits	Supporting evidence
Diet (Mediterranean, MIND, and omega-3)	Reduces neuroinflammation, oxidative stress, and beta-amyloid plaque buildup	Studies show cognitive benefits and reduced risk of AD
Physical exercise	Enhances neuroplasticity and improves motor function	Clinical trials confirm cognitive and motor benefits in AD and PD patients
Sleep quality	Supports memory consolidation and reduces beta-amyloid accumulation	Sleep deprivation linked to increased neurodegeneration
Cognitive training	Improves memory, executive function, and problem-solving skills	Studies show delayed cognitive decline and increased brain activity
Stress management (Yoga, meditation, and CBT)	Reduces cortisol levels, improves mood, and enhances cognitive function	Proven to lower stress-related neurodegeneration
Social engagement	Lowers risk of dementia, maintains brain connectivity, and improves emotional well-being	Research indicates social isolation increases neurodegeneration risk

Abbreviations: AD: Alzheimer's disease; CBT: Cognitive behavioral therapy; MIND: Mediterranean-Dietary Approaches to Stop Hypertension Diet Intervention for Neurodegenerative Delay; PD: Parkinson's disease.

is the lack of effectiveness. The impact of lifestyle changes, CT, and physical exercise varies widely among individuals, depending on factors such as genetics, disease severity, and personal commitment.[8] Unlike medications, which have standardized dosages and mechanisms, NPIs lack uniform protocols, making it difficult to predict outcomes.[9] Another limitation is that many NPIs, such as exercise regimens and dietary modifications, require long-term commitment, which can be challenging for patients experiencing cognitive decline or mobility issues.[10] In addition, some interventions are costly and not widely available, limiting access for many patients.[11] Moreover, the scientific evidence supporting NPIs remains incomplete. While studies suggest benefits, long-term clinical trials comparing NPIs directly to pharmacological treatments are limited. Many findings are based on small-scale or observational studies, making it difficult to draw definitive conclusions about their role in slowing or preventing neurodegeneration.[12] Despite these challenges, NPIs remain a promising complementary approach, especially when combined with conventional medical treatments. However, further research is needed to establish standardized guidelines and improve accessibility for a wider population.

6. Conclusion

The increasing prevalence of NDs among the aging population has intensified the focus and effort on tackling this phenomenon both pharmacologically and through non-pharmacological means. Given the limitations of many drugs, including issues with transport and absorption processes, as well as overall efficacy, the pursuit to uncover the potential of NPIs has clinically revealed the mounting benefits and true effectiveness. Research has proven that NPIs, such as nutrition, resistance exercise, cognitive stimulation, quality sleep, and early screening, can mitigate the pathological signs of NDs and slow their progression, especially in the early stages. However, due to the heterogeneity and severity of such NDs, there is no conclusive evidence to suggest that NPIs can truly substitute the benefits of conventional treatment. Hence, this warrants further research and precise conduction of large-size clinical trials in multiple communities. Current research gives a clear insight that the combined treatment has remarkable benefits and ameliorates the symptoms of neurodegeneration, aiming at a better quality of life for patients.

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ACCSCIENCE PUBLISHING

Advanced Neurology

REVIEW ARTICLE

Neurotoxicity of lisdexamfetamine: Implications for neuronal health and central nervous system function

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Abstract

Lisdexamfetamine (LDX) is a d-amphetamine prodrug with a long-acting therapeutic profile. It has become well-known in recent years due to its widespread use in treating several psychological disorders, such as attention-deficit/hyperactivity disorder and binge eating disorder. However, concerns have been raised about its potential neurotoxic properties, particularly with long-term use. Although direct evidence of LDX-induced neurotoxicity is limited, insights can be drawn from studies on the harmful impacts of its parent compound and related amphetamines, such as amphetamine and methamphetamine, on the central nervous system. The potential mechanisms through which these drugs exert their neurotoxic effects include mitochondrial dysfunction, oxidative stress, neuroinflammation, synaptic failure, and excitotoxicity, all of which contribute to neuronal injury and death. Furthermore, amphetamines have been shown to disrupt the blood–brain barrier, likely triggered by the aforementioned mechanisms, with neuroinflammation being the most significant factor. In this review, we aim to synthesize the available knowledge on the potential mechanisms behind LDX-induced neurotoxicity and emphasize the need for future studies to better understand the long-term side effects of LDX.

Keywords: Lisdexamfetamine; Central nervous system; Neuroinflammation; Attention-deficit/hyperactivity disorder

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1. Introduction

Lisdexamfetamine (LDX) is a prodrug of the central nervous system (CNS) stimulant substance d-amphetamine. It is the first chemically manufactured stimulant prodrug that offers a novel long-acting medication for several psychological conditions.[1] The most prominent advantage of LDX over other formulations is that, as a prodrug, it does not contain the active form of the drug, which could be altered during ingestion and absorption in the gastrointestinal tract. However, LDX has a complex drug delivery mechanism that involves the enzymatic hydrolysis of the prodrug to active d-amphetamine and the naturally occurring amino acid L-lysine.[2] Since 2007, LDX has been approved for treating attention-deficit/hyperactivity disorder (ADHD) in children aged 6 – 12 years, and in April 2008, the United States Food and Drug Administration

approved its use in adults as well.[3] In addition to ADHD, LDX is also used for treating binge eating disorder (BED).[4]

Amphetamine has two enantiomers: Levoamphetamine (l-amphetamine) and dextroamphetamine (d-amphetamine). D-amphetamine is a non-catecholamine sympathomimetic amine known for its CNS-stimulating properties. It blocks several critical neurotransmitter transporters at the synaptic cleft, including dopamine transporter, noradrenaline transporter, and vesicular monoamine transporter 2. It can also block the serotonin transporter, but with lower affinity. Furthermore, it functions as a mild monoamine oxidase (MAO) inhibitor.[5] In norepinephrine neurons, d-amphetamine is a far more potent catecholamine uptake inhibitor than l-amphetamine. However, both isomers are equally effective in inhibiting catecholamine uptake in dopamine neurons in the corpus striatum. D-amphetamine ultimately inhibits the absorption of catecholamines (i.e., norepinephrine and dopamine) into the presynaptic neuron, increasing their availability in the synaptic space.[1] The specific mechanism of action of LDX in ADHD and BED has not been fully explored; nonetheless, the clinical effects of LDX are thought to be linked to the pharmacological activities of d-amphetamine.[5] Human pharmacokinetic investigations have revealed that d-amphetamine exposure following oral LDX administration is monophasic, persistent, and dose-proportional, with minimal variability both within one patient and between patients. This facilitates dose optimization.[6] In addition, because LDX does not impact cytochrome P450 enzymes, it has a minimal risk of pharmacokinetic drug–drug interactions.[7]

Like any other medication, LDX has been found to induce a wide range of side effects. The adverse effects associated with LDX are similar to those expected with other stimulants. The most prevalent side events after LDX administration in children, adolescents, and adults with ADHD include anxiety, irritability, insomnia, anorexia, weight loss, nausea, vomiting, upper abdominal discomfort, diarrhea, dizziness, and dry mouth.[8] Similarly, the most common side effects of this drug in individuals with BED include dry mouth, insomnia, jitteriness, elevated heart rate, decreased appetite, anxiety, and constipation.[8] Furthermore, LDX has been shown to be prone to abuse in some cases.[9] However, in abuse liability studies involving individuals with a history of stimulant dependence, LDX is linked to lower abuse liability responses compared to immediate-release dexamfetamine (Table 1).[10]

As previously stated, once converted into its active form, d-amphetamine, LDX significantly affects neurotransmitter dynamics in the brain, mainly by increasing dopamine and norepinephrine levels. However, it can be argued that prolonged and excessive activation of these pathways may lead to neurotoxicity. This argument is supported by several animal studies showing that chronic exposure to high doses of amphetamine, the active form of LDX, can impair synaptic plasticity and cause cognitive deficits.[14,15] These findings emphasize the need for further research into the long-term effects of LDX on neurons and other CNS cell lines. Accordingly, this review carefully examines the available evidence regarding LDX's potential neurotoxic effects, with an emphasis on neuronal health.

Table 1. Common adverse events (AEs) reported for lisdexamfetamine

Study	Sample	Common AEs	Severity	Onset time
Biederman *et al.*[11]	Children (*n*=52)	Insomnia, appetite loss	Mild to moderate	Week 1
Wigal *et al.*[12]	Children (*n*=129)	Irritability, headache	Mild	Early treatment
Weisler *et al.*[13]	Adults (*n*=191)	Respiratory infection, insomnia	Mild-to-severe	1st week

Table 2. Structure and activation pathway of lisdexamfetamine

Stage	Location	Conversion/action
Absorption	Small intestine	Absorbed via PEPT1
Hydrolysis	Red blood cells	Converted to d-amphetamine and L-lysine
Pharmacological effect	Brain	Inhibits reuptake and promotes release of DA/NE

Abbreviations: DA: Dopamine; NE: Norepinephrine; PEPT1: Peptide transporter 1.

2. Mechanism of LDX's action in the CNS

LDX is a prodrug composed of a peptide bond covalently linking d-amphetamine to the naturally occurring amino acid L-lysine, rendering the parent molecule pharmacologically inactive. After oral administration, LDX is actively absorbed in the small intestine via peptide transporter 1.[16] Studies have indicated that LDX is hydrolyzed in human blood, with bioconversion specifically occurring in red blood cells (RBCs).[16] The rate of LDX hydrolysis is hematocrit-dependent. However, significant conversion to d-amphetamine still occurs even when hematocrit levels are reduced to 10% or 25% of normal.[16] Moreover, the hydrolysis rate remains unchanged even when RBCs undergo lysis,[16] and the release of d-amphetamine from LDX is unaffected in blood from donors with defective RBCs caused by sickle cell disease (Table 2).[17]

LDX has been shown to enhance impulse control in individuals with BED and ADHD.[18,19] It is believed that, like

other psychostimulants, LDX improves impulse control by modulating the prefrontal cortex (PFC), a brain area involved in self-regulation and inhibitory control.[20] LDX influences the pharmacological activity of seven human proteins. Its active form stimulates the CNS by inhibiting the activity of the synaptic vesicular amine transporter (encoded by *SLC18A2*), dopamine transporter (encoded by *SLC6A3*), noradrenaline transporter (encoded by *SLC6A2*), and serotonin transporter (encoded by *SLC6A4*). All of these targets are involved in presynaptic signaling, suggesting that LDX regulates three major neurotransmitter pathways: Dopamine, norepinephrine, and serotonin.[21] In addition, LDX activates the trace amine-associated receptor 1, which may help attenuate several maladaptive eating behaviors. It also functions as a neuro-maintenance agent by inhibiting oxidative enzymes MAO-A and MAO-B (Table 3).[21] Furthermore, LDX has been reported to modulate several inflammatory mediators.[22] Tumor necrosis factor (TNF) is induced following LDX treatment, suggesting a potential immunomodulatory role of the drug. Other cytokines stimulated by LDX include interleukin (IL)-4, IL-6, and IL-10. Notably, IL-4 and especially IL-10 exhibit anti-inflammatory properties, indicating that LDX may contribute to the regulation of the inflammatory response (Table 4).[21]

Table 3. Biochemical pathways involved in lisdexamfetamine (LDX) action

Pathway	Proteins/targets	Effect of LDX
Dopaminergic	DAT, VMAT2, MAO-A	Inhibits reuptake and degradation
Noradrenergic	NET	Inhibits reuptake
Serotonergic	SERT	Weak inhibition of reuptake
Immunomodulatory	Tumor necrosis factor -α, IL-4, IL-10	Modulates inflammatory response
Receptor-mediated	TAAR1	Regulates compulsive behavior

Abbreviations: DAT: Dopamine transporter; MAO: Monoamine oxidase A; NET: Noradrenaline transporter; SERT: Serotonin transporter; TAAR1: Trace amine-associated receptor 1; VMAT2: Vesicular monoamine transporter 2.

Table 4. Immune and inflammatory mediators stimulated by lisdexamfetamine (LDX)

Mediator	Immune role	Status with LDX
TNF-α	Pro-inflammatory	Increased
IL-1β	Pro-inflammatory	Possibly increased
IL-6	Dual role	Upregulated
IL-10	Anti-inflammatory	Mildly increased
IL-4	Anti-inflammatory	Mildly increased

Abbreviations: IL: Interleukin; TNF: Tumor necrosis factor.

One important consideration is that these mechanisms, and how each patient responds to LDX, may be further complicated by individual genetic differences. Therefore, pharmacogenomics studies are essential to clarify how LDX leads to improved outcomes or is better tolerated in specific genotypes.[23] To summarize, the pharmacological profile of LDX as a prodrug is complex: It must undergo hydrolysis to convert into its active form, d-amphetamine, which significantly affects neurotransmitter systems, particularly dopamine and norepinephrine. The mechanism of LDX action involves multiple proteins, such as neurotransmitter transporters and oxidative enzymes. Additionally, its modulation of inflammatory mediators, including TNF and IL-10, suggests a potential immunomodulatory role. However, the exact molecular mechanisms of LDX action, particularly how individual genotypes influence drug response, remain unclear.

3. Adverse events (AEs) associated with LDX

Numerous clinical trials have investigated the safety and efficacy of LDX, consistently identifying several common AEs associated with its usage. For instance, Biederman *et al.*[24] conducted a randomized crossover study comparing the efficacy of LDX and mixed amphetamine salts extended-release (MAS-XR) in a simulated classroom setting involving 52 children with ADHD aged 6 – 12 years. In the LDX group, the most frequently reported AEs were insomnia, decreased appetite, and anorexia, whereas MAS-XR was linked to reduced appetite, stomach discomfort, vomiting, and insomnia. In another large multicenter trial, Biederman *et al.*[11] studied 290 children with ADHD and found that over 95% of all AEs were mild to moderate, most occurring within the 1st week of treatment. Common AEs in this study included reduced appetite, insomnia, irritability, stomach discomfort, and headache. Similarly, Wigal *et al.*[12] conducted a randomized, placebo-controlled crossover study in 129 children, where the most commonly reported AEs were mood swings, irritability, headache, and loss of appetite. These findings are consistent with a longitudinal research by Findling *et al.*[25] which monitored 272 children treated with LDX over 12 months. The most frequent AEs, such as weight loss, reduced appetite, and insomnia, occurred mostly during the 1st month of therapy, with no significant AEs specifically attributed to LDX.

To summarize, the most common AEs reported in LDX clinical research across both pediatric and adult populations are reduced appetite, insomnia, headache, and irritability. These side effects typically emerge early in therapy and tend to diminish over time. The potential neurotoxicity of LDX may be linked to the same mechanisms that underlie these AEs, including overstimulation of dopaminergic and

norepinephrine pathways. Long-term exposure may also induce oxidative stress and neuroinflammation, raising concerns regarding its continued usage, particularly in vulnerable populations such as children and individuals with pre-existing conditions. Although the temporal pattern of AE development does not align with this theory, as most AEs appear early, evidence on long-term exposure remains scarce. Therefore, additional longitudinal studies are necessary to gain a more comprehensive understanding of these risks.

4. LDX-associated neurotoxicity

LDX is associated with potential neurotoxic effects on the CNS. While direct information on the mechanisms of LDX-induced neurotoxicity is limited, it is critical to examine the broader class of stimulant medications to which it belongs, as it is a prodrug of d-amphetamine. This class includes amphetamine and methamphetamine, both of which have well-documented neurotoxic effects. Previous studies have shown that chronic exposure to stimulants can cause oxidative stress, mitochondrial malfunction, and an imbalance between excitatory and inhibitory neurotransmission. In addition, preclinical and clinical investigations have identified neuroinflammation and blood–brain barrier (BBB) disruption, raising concerns regarding the long-term safety of LDX. Together, these factors create a neurotoxic environment that can lead to neuronal death and will be discussed in detail below.

4.1. Oxidative stress and mitochondrial dysfunction

Oxidative stress is a key factor in amphetamine-induced neurotoxicity.[26] Given LDX's structural similarity to amphetamine, it is plausible to assume similar mechanisms are involved. Administering a high dose of amphetamine and its analogs has been shown to increase free radical production, while pretreatment with antioxidants reduces dopaminergic deficits caused by amphetamine.[27] Furthermore, previous studies have reported that overexpression of superoxide dismutase, a free radical scavenger, inhibits the neuronal impairments caused by methamphetamine.[28] Taken together, these findings suggest that the formation of reactive oxygen species (ROS) mediates the harmful effects of amphetamine administration. However, amphetamines can induce oxidative stress via several distinct pathways.

Research has revealed that amphetamine and methamphetamine significantly influence mitochondrial function.[29] Under normal physiological conditions, mitochondria are the main source of intracellular ROS.[30] ROS are generated by the electron transport chain complexes on the inner mitochondrial membrane and by monoamine oxidase on the outer membrane. Therefore, one of the primary mechanisms through which amphetamines may increase ROS production is by changing basal mitochondrial activities.[31]

In addition, given that amphetamines promote dopamine release, another probable source of ROS is dopamine autoxidation through the Fenton reaction, which employs iron as a cofactor.[32] It has also been proposed that increased intracellular dopamine levels may result in the formation of reactive dopamine quinones,[32] which subsequently produce ROS. A third proposed mechanism involves calcium homeostasis. Amphetamines elevate intracellular calcium levels and activate nitric oxide synthase, leading to increased production of reactive nitrogen species like peroxynitrite, which may further contribute to oxidative stress.[27]

As a result of these mechanisms, reactive chemicals are produced and subsequently oxidized, ultimately disrupting the function of various cellular components such as proteins, lipids, and nucleic acids. This oxidative damage may also result in microglial activation.[33] Excessive stimulation of microglia appears to contribute to some of the neurotoxic effects of amphetamines, including the degradation of dopaminergic nerve endings.[34] Furthermore, microglial activation is often followed by astroglial activation, which leads to elevated levels of pro-inflammatory cytokines, as discussed in the next section (Tables 5 and 6).[33,35]

In summary, studies on amphetamines indicate that oxidative stress and mitochondrial dysfunction are key

Table 5. Mechanisms of action underlying lisdexamfetamine-associated neurotoxicity

Mechanism	Description
Dopamine release	Enhances dopamine release and inhibits its reuptake, increasing synaptic dopamine levels
Norepinephrine modulation	Blocks norepinephrine reuptake and increases its release, enhancing adrenergic activity
Serotonin interaction	Although not primary, LDX may indirectly affect serotonin systems, especially at high doses.
Excitotoxicity potential	Elevated glutamate release linked to d-amphetamine may contribute to neuronal excitotoxicity
Oxidative stress induction	Stimulant-induced ROS generation can damage neuronal structures and functions
Mitochondrial dysfunction	Oxidative stress may impair mitochondrial function and energy metabolism
Neuroinflammation	Stimulant-induced activation of microglia and cytokine release may promote neuroinflammation
BBB disruption	LDX, like other amphetamines, may compromise BBB integrity under certain conditions

Abbreviations: BBB: Blood–brain barrier; LDX: Lisdexamfetamine.

Table 6. Possible sources of reactive oxygen species production induced by lisdexamfetamine

Source	Mechanism
Mitochondrial activity	• Electron transport chain dysfunction • MAO-mediated dopamine metabolism
Dopamine autoxidation	Iron-dependent Fenton reaction leading to dopamine-quinone formation
Intracellular Ca^{2+} rise	NOS activation and RNS production

Abbreviations: Ca^{2+}: Calcium ions; MAO: Monoamine oxidase; NOS: Nitric oxide synthase; RNS: Reactive nitrogen species.

contributors in drug-induced neurotoxicity. Although direct evidence for LDX is lacking, its potential effects should be considered in light of findings from related compounds.

4.2. Neuroinflammation and immune response activation

Autoimmunity, which is strongly associated with oxidative stress, also plays an important role in amphetamine-induced neuronal damage. Neuroinflammation can disrupt neurobiological pathways that regulate cognitive processes, such as inhibiting long-term potentiation and synaptic plasticity, while also dysregulating several metabolic processes, such as the tryptophan-kynurenine pathway, neurotrophin metabolism, and the hypothalamic–pituitary–adrenal axis.[36] Several studies have shown that the stimulant drug methamphetamine increases levels of at least one classic pro-inflammatory cytokine, including TNF-α, IL-1β, and IL-6, in various brain regions, such as the PFC, nucleus accumbens, ventral tegmental area, hypothalamus, and striatum.[37-40]

However, other studies found that these cytokines are not significantly altered in the hippocampus, PFC, or nucleus accumbens after a single administration of stimulants (amphetamine and methamphetamine).[41] Several other studies report asymmetric results across brain regions,[37,42,43] suggesting that drug-induced cytokine alterations are likely region-dependent and primarily affect dopaminergic neurons. It is also worth noting that additional factors, such as dosage, administration methods, treatment duration, and cytokine quantification methodology, may explain discrepancies across studies. For instance, dosage appears to play a significant role in this heterogeneity, with studies using low doses of stimulants generally showing no changes in cytokine levels,[43,44] whereas higher doses are associated with increased levels of classic cytokines.[37-40]

Although *in vivo* investigations on the pro-inflammatory effects of LDX are limited, a recent *in silico* study has provided some insight. Using a systems biology-based virtual model of LDX, researchers investigated the molecular pathways affected by the drug.[45] This model suggests that LDX-induced impairment of dopamine signaling may regulate neuroinflammation by influencing various molecular targets, including cytokines such as TNF-α, IL-6, IL-1B, and interferon gamma receptors, as well as immune cells including microglia, T cells, and monocytic cells.

In addition to increasing the expression of pro-inflammatory cytokines, psychostimulants contribute to the development of neuroinflammatory processes by disrupting the structure and function of glial cells, leading to pathological events such as microgliosis and astrogliosis. For instance, microglial hypertrophy has been reported as a pronounced consequence of amphetamine exposure in the hippocampus of mice.[33] However, unlike pro-inflammatory cytokines, some brain areas rich in dopaminergic neuron bodies, such as ventral tegmental area and substantia nigra, have low sensitivity to amphetamine-induced microglial activation.[33] Additionally, chronic methamphetamine exposure has been shown to cause significant microglial activation, as evidenced by increased expression of ionized calcium-binding adapter molecule 1, a microglial activation marker, in the striatum and hippocampus of mice.[44]

Furthermore, substantial data suggest that both human amphetamine users and rats exposed to amphetamine exhibit diminished neuronal integrity and impaired glial responsiveness in the PFC.[33,46,47] In addition, methylphenidate, another widely prescribed psychostimulant, has been shown to induce widespread microglial activation with chronic exposure. In a study by Carias *et al.*,[48] such activation was observed across several brain regions, such as the somatosensory and motor cortices, insular cortex, thalamus, hippocampus, substantia nigra, and globus pallidus, both shortly after treatment initiation and even after its cessation. These findings indicate that similar mechanisms of glial activation may occur with LDX. However, as a prodrug, LDX may produce a more complex pattern of amphetamine-induced glial reactivity, necessitating further investigation.

In summary, neuroinflammation triggered by amphetamines, and likely LDX, underscores the drug's possible neurotoxicity. Although direct evidence remains limited, the pharmacological similarities among these compounds highlight the need for additional investigation into LDX's role in promoting neuroinflammation and immune response activation.

4.3. Excitotoxicity and neurotransmitter imbalance

Excitotoxicity refers to cell death caused by the detrimental effects of excitatory amino acids. Neuronal excitotoxicity often involves the damage and death of neurons due to

prolonged exposure to glutamate, the principal excitatory neurotransmitter in the CNS, resulting in excessive ion influx into the cell.[49] Consequently, the role of excitotoxicity in the neurotoxic mechanism of amphetamines may be ascribed to an amphetamine-induced elevation in striatal glutamate release. This is supported by evidence showing that the use of both metabotropic (e.g., 2-methyl-6-[phenylethynyl]-pyridine) and ionotropic (e.g., MK-801) glutamate receptor antagonists can mitigate the neurotoxicity caused by amphetamine and methamphetamine.[29]

As previously mentioned, d-amphetamine functions by inhibiting the reuptake of norepinephrine and dopamine into the presynaptic neuron while enhancing the release of these monoamines into the synaptic space. Although this mechanism contributes to the therapeutic effects of LDX at optimal doses and short-term exposure, its influence on neurotransmitter homeostasis at elevated doses and over prolonged periods remains poorly understood. Numerous animal studies have demonstrated that methamphetamine, which possesses a nearly identical chemical structure to d-amphetamine, can impair both the dopamine and serotonin systems.[50-52]

Furthermore, methamphetamine toxicity, especially following repeated high-dose administration, selectively affects specific neural systems, notably those within the limbic reward system (e.g., substantia nigra, striatum, and nucleus accumbens).[53] Chronic methamphetamine use may also diminish dopamine levels, receptor density, and dopamine transporter expressions.[52] Notably, postmortem studies have indicated that even recreational doses of methamphetamine can lead to substantial dopamine depletion.[54]

Overall, the pharmacological and structural similarities between LDX and other amphetamines suggest that excitotoxicity could be a concern with LDX usage. However, concrete proof is necessary. Further research is essential to clarify LDX's influence on neurotransmitter systems, particularly in the context of drug misuse.

4.4. Disruption of the BBB

Amphetamines are known to compromise the integrity of the BBB, and similar issues may apply to LDX. The BBB is a specialized structure composed of tightly connected brain endothelial cells that form a boundary between the CNS and the rest of the body.[55] Once considered static, the BBB is now recognized as a dynamic interface that responds to signals from various sources. Recent data indicate that drugs of abuse, such as stimulants, can impair its functions.[56] Hyperthermia and hypertension induced by high doses of amphetamines are primary causes of transient BBB disruptions, leading to localized neurodegeneration and neuroinflammation in laboratory animals.[57] In addition, alterations in tight junction complexes have been demonstrated to impair the BBB integrity following methamphetamine administration. Methamphetamine exposure has been shown to reduce the expression of tight junction proteins such as zona occluden 1, occludin, and claudin 5 in both *in vivo* and *in vitro* studies.[58-62] Ultimately, neuroinflammation is proposed as a key mechanism underpinning methamphetamine-induced neurotoxicity and may also contribute to BBB damage.[56]

In summary, the breakdown of the BBB by amphetamines raises concerns about similar effects with LDX. Given that the BBB is essential for maintaining brain homeostasis and neuronal viability, further research is required to ascertain whether LDX can also disrupt its integrity.

5. Discussion

The current evidence suggests a potential association between LDX use and neuropsychological pathologies, which can be categorized into two distinct groups: Early-onset side effects and long-term neurotoxic effects. Although early-onset side effects, including insomnia, appetite suppression, headache, and irritability, are observed shortly after LDX use, it is important to differentiate whether these non-specific symptoms are typical of general stimulant side effects or indicative of underlying neurotoxicity.[63] In contrast, the expected long-term neurotoxic effects, such as oxidative stress, neuroinflammation, and excitotoxicity, are believed to develop gradually and accumulate with increasing exposure.[64]

The temporal gap between early-onset side effects and long-term neurotoxicity raises significant questions about their underlying mechanisms. Some early side effects may reflect acute dopaminergic and noradrenergic overstimulation, which could also contribute to neurotoxicity under chronic conditions.[65,66] Although the pharmacokinetic profile of LDX, as a prodrug that produces a consistent release of d-amphetamine, may help reduce the intensity of such overstimulation in the short term, it could lead to cumulative stress on neural systems over time.[67] However, due to the lack of mechanistic studies investigating both acute and chronic effects within the same experimental framework, no definite link can yet be established. Therefore, future studies should explore whether early behavioral symptoms can serve as biomarkers or early indicators of long-term neuronal damage.

Another significant gap in current knowledge is that, although several neurotoxic mechanisms have been proposed in relation to amphetamine-related compounds, no study to date has specifically examined the temporal sequence or hierarchical contribution of these pathways.

Nonetheless, current preclinical and mechanistic data suggest an interconnected and synergistic network rather than separate pathways operating independently. For instance, while neuroinflammation may itself prolong excitotoxic damage and affect the integrity of the BBB, oxidative stress could act as an upstream trigger, amplifying mitochondrial dysfunction and initiating neuroinflammation.[68] These connections suggest a complex neurotoxic environment where several pathways likely cooperate. Future experimental research is needed to clarify the temporal and causal links among these processes, which could inform both the prediction and prevention of LDX-related neurotoxicity.

Another significant aspect that has been inadequately addressed in earlier studies is the dose-dependent nature of LDX-related effects. With limited research on the dose-dependent long-term effects, human trials of LDX have primarily focused on its efficacy and common side effects.[69,70] While the possibility for cumulative effects at higher or prolonged exposures remains unquantified, LDX gradually delivers d-amphetamine through enzymatic conversion, making it plausible that its pharmacokinetic profile mitigates acute neurotoxic effects at recommended doses. Future research should systematically evaluate dose-response interactions utilizing both neurochemical and functional endpoints across different treatment durations to strengthen risk assessments and inform therapeutic decision-making.

Finally, there are limitations of this study that should be noted. First, as very few clinical trials directly assess the long-term neurotoxicity of LDX in human subjects, the present evidence base on LDX-induced neurotoxicity is essentially derived from preclinical studies on amphetamine and methamphetamine. Although these compounds are pharmacologically linked to LDX's active metabolite (d-amphetamine), they may not entirely reflect the drug's pharmacokinetics and tissue-specific effects. Second, this review utilizes results from various experimental contexts, including different dosages, animal models, and modes of administration, which may limit the generalizability of the findings. Lastly, the lack of longitudinal human studies prevents any definitive conclusions regarding the degree of risk or causality. These limitations emphasize the need for future translational and clinical research to more precisely define the safety profile of LDX.

Despite the potential neurotoxic impacts reviewed in this paper, LDX remains a clinically valuable pharmacological option for treating ADHD and BED.[4,71] It provides a long-acting, well-tolerated substitute with a reduced risk of abuse due to its prodrug design.[72] Therefore, the neurotoxic hazards of this drug should be considered not in isolation, but in line with its therapeutic advantages.

6. Conclusion

The neurotoxic potential of LDX is increasingly concerning, primarily due to its structural resemblance to amphetamines, which are known to cause oxidative stress, mitochondrial dysfunction, excitotoxicity, neuroinflammation, and impairment of the BBB. While direct studies on the long-term effects of LDX are limited, findings from research on other stimulants, such as amphetamines and methamphetamines, indicate that prolonged exposure to LDX may pose significant risks to neuronal health and CNS function.

7. Future directions

Future research must address the significant gaps in our understanding of LDX-induced neurotoxicity. Comprehensive longitudinal studies are essential to assess the long-term effects of LDX on the brain, with particular focus on oxidative stress, mitochondrial integrity, and synaptic plasticity. The studies should evaluate the drug's effects on neuronal survival, cognitive function, and behavioral outcomes in both preclinical and clinical settings.

Furthermore, investigating the mechanisms through which LDX influences the BBB and immune response is critical. Understanding the impact of prolonged LDX usage on neuroimmune interactions and its role in neuroinflammation may help reduce potential CNS injuries in vulnerable populations. Moreover, pharmacogenomic research may elucidate how genetic variants influence patient responses to LDX, thereby informing personalized treatment strategies to mitigate neurotoxic risks.

Striking a balance between the therapeutic benefits of LDX and its long-term safety is crucial. Enhancing our knowledge of the drug's neurobiological effects will enable clinicians to make more informed treatment decisions, ensuring that LDX remains a viable and safe option for patients.

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REVIEW ARTICLE

Facilitating recovery from post-stroke depression with acupuncture: A comprehensive analysis of systematic reviews

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Abstract

Post-stroke depression (PSD) is the most common psychological complication among stroke survivors and is strongly associated with poor outcomes and increased mortality. Acupuncture has been proposed as a potential treatment for PSD. In this overview, 13 meta-analyses (MAs) of randomized controlled trials published up to August 2023 that evaluated acupuncture for PSD were identified. Across these MAs, acupuncture generally demonstrated greater efficacy than control interventions in alleviating depressive symptoms. However, methodological assessment using the A MeaSurement Tool to Assess Systematic Reviews 2 (AMSTAR-2) rated all included studies as having critically low quality, indicating pervasive design and reporting weaknesses. Reporting quality, as evaluated by the Preferred Reporting Items for Systematic Reviews and MAs guidelines, was relatively high, with over 90% of checklist items being rated "yes" or "partially yes." When the quality of evidence was appraised through the grading of recommendations assessment, development, and evaluation system, only nine of 61 outcomes were classified as moderate quality, 29 as low quality, and the remainder as very low quality. Although acupuncture appears to be a safe and potentially beneficial option for managing PSD, the overall strength of evidence remains limited. Thus, these findings should be interpreted with caution. Future research should prioritize rigorous trial design, standardized intervention protocols, and comprehensive meta-analytic methods to enhance the reliability and clinical applicability of evidence on acupuncture for PSD.

Keywords: Acupuncture; Overview; Post-stroke depression; Systematic review; AMSTAR-2; GRADE; PRISMA; Treatment

1. Introduction

Stroke is a prevalent cerebrovascular disease marked by high incidence, mortality, and disability rates.[1] Among its sequelae, post-stroke depression (PSD) represents the most common psychiatric complication, affecting approximately one-third of survivors, according to epidemiological data.[2,3] As the global population continues to age, the prevalence of PSD is expected to increase, imposing a substantial societal and healthcare burden.[1] Clinically, PSD is associated with impaired physical and cognitive rehabilitation, suboptimal functional recovery, and reduced quality of life, often hindering patients' participation in rehabilitation programs.[4] Moreover, individuals with PSD exhibit higher risks of self-harm or suicidal ideation[5,6] and are more susceptible to recurrent cerebrovascular events – factors that contribute to poorer prognoses and elevated mortality rates.[7,8]

Present PSD management typically involves pharmacological and non-pharmacological approaches. Antidepressant medications – including monoamine oxidase inhibitors, selective serotonin reuptake inhibitors, and tricyclic antidepressants – constitute the standard pharmacotherapy. Nevertheless, the efficacy of these agents remains contested, and their use can be accompanied by adverse effects.[9,10] As a result, non-pharmacological interventions have been adopted to mitigate side effects and enhance overall therapeutic outcomes.

Acupuncture, as a prominent non-pharmacological modality, has demonstrated therapeutic benefits in PSD. Compared with pharmacotherapy alone, acupuncture is cost-effective, easy to administer, and associated with minimal side effects.[11] Existing studies suggest that combining acupuncture with conventional antidepressants yields superior efficacy, faster onset of action, and favorable tolerability.[12] Given the growing body of literature – often yielding inconsistent results – this overview aims to systematically synthesize and critically appraise high-quality meta-analyses (MAs) of randomized controlled trials (RCTs) on acupuncture for PSD, with the goal of informing future research directions and supporting evidence-based clinical decision-making.

2. Methods

This study systematically searched for MAs of acupuncture therapy for post-stroke functional impairments, encompassing literature from the databases' inception up to August 20, 2023. Seven databases were searched, including Cochrane Library, Embase, PubMed, China National Knowledge Infrastructure, Wanfang Database, China Science and Technology Journal Database, and Chinese Biomedical Literature Database. No restrictions were imposed on publication dates or language.

The search strategy was developed based on a combination of subject headings and free-text terms, focusing on keywords, such as "acupuncture," "stroke," and "systematic review." Adjustments to the search strategy were made according to the unique features of each database. In addition, to ensure comprehensive data collection, the reference lists of relevant reviews were manually searched to identify additional eligible studies.

This protocol has been successfully registered on and can be accessed through PROSPERO (https://www.crd.york.ac.uk/prospero/, ID: CRD42020189173).

2.1. Inclusion criteria

Two reviewers (PL and LW) independently evaluated the eligibility of studies according to the following criteria:

(i). Studies included in MAs were limited to RCTs.

(ii). Study participants were diagnosed following internationally recognized guidelines or consensus, with no restrictions on the version of guidelines used, and there were no limitations based on gender, age, race, disease duration, severity, or source.

(iii). Types of acupuncture included all invasive acupuncture methods, such as acupuncture with needles, including various needle stimulation techniques (e.g., electroacupuncture, manual acupuncture, or warm needling). Non-invasive acupuncture techniques, such as laser acupuncture, ear acupressure pellets, transcutaneous electrical nerve stimulation, and acupoint massage, were excluded. The experimental group comprised acupuncture or acupuncture combined with other conventional treatments, while the control interventions included placebo, sham, or conventional treatments. Antidepressants used in conventional treatments include monoamine oxidase inhibitors, tricyclic antidepressants, tetracyclic antidepressants, selective serotonin reuptake inhibitors for PSD, and other related agents.

(iv). Outcome measures were defined in accordance with internationally recognized clinical trial guidelines and scales. Primary outcomes were focused on the clinical overall effective rate and improvement in functional scale scores, while secondary outcomes included adverse events and adverse reactions.

2.2. Exclusion criteria

The following exclusion criteria were applied:

(i). Duplicate publications or studies published multiple times.

(ii). Participants diagnosed with PSD along with other concurrent medical conditions.

(iii). Studies in which acupuncture was employed as an intervention in the control group.

(iv). Studies where acupuncture was not the sole distinguishing variable between the intervention and control groups.
(v). Studies for which the full text was unavailable for review.
(vi). Studies categorized as opinion articles, evidence summaries, guidelines, editorials, conference abstracts, or those with incomplete or non-extractable data.

2.3. Research selection

The studies retrieved from the search were imported into EndNote X9.3.3 (Clarivate, US). After removing duplicates, two independent researchers (PL and LW) screened the studies based on the inclusion and exclusion criteria. Initial screening was performed by reviewing titles and abstracts, followed by a second screening involving a thorough review of the full texts. Detailed records were kept to document the reasons for excluding studies during the second screening phase. The final selection results were cross-checked by both researchers (PL and LW), and any discrepancies regarding the inclusion of individual studies were resolved through discussion. In cases where consensus could not be reached through discussion, the third researcher (AW) intervened.

2.4. Data extraction

Two reviewers (PL and LW) independently extracted data from the included MAs using a pre-designed table. This table included sections for basic information (e.g., first author, publication year, and information source), methods (e.g., searched databases, number of included RCTs, and sample sizes), study design (e.g., diagnostic criteria, interventions, control treatments, and tools used to assess methodological quality), and outcomes (e.g., main conclusions, safety, and quality outcomes). Discrepancies related to the extracted data were resolved through discussion. In cases where consensus could not be reached through discussion, the third researcher (AW) intervened.

2.5. Quality assessment

2.5.1. Methodological quality

Two researchers (PL and LW) independently used the A MeaSurement Tool to Assess Systematic Reviews 2 (AMSTAR-2)[13] to assess the methodological quality of MAs. AMSTAR-2 comprises 16 items for evaluating the effectiveness of MAs, with seven items (Items 2, 4, 7, 9, 11, 13, and 15) considered crucial for assessing the effectiveness of MAs. Each item has three possible responses: "yes," "partial yes," or "no." Methodological quality is rated as "high" when there are no critical weaknesses or only one non-critical weakness; "moderate" when there is more than one non-critical weakness but no critical flaws; "low" when one critical flaw and numerous non-critical weaknesses exist; and "very low" when there are more than one serious flaw. During the assessment process, any discrepancies were resolved through discussion.

2.5.2. Report quality

Two researchers (PL and LW) independently utilized the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) tool[14] to assess the quality of MAs' reports. PRISMA consists of 27 items that encompass seven aspects of MAs, including titles, abstracts, introductions, methods, results, discussions, and other information. Each item in PRISMA was assessed as "yes," "partial yes," or "no" based on established criteria. In case of any discrepancies, they were resolved through discussion.

2.5.3. Evidence quality

In the context of MA, the evidence quality for each outcome measure was independently assessed by two authors (PL and LW) using the grading of recommendations assessment, development, and evaluation (GRADE) methodology.[15] GRADE ranks evidence based on factors, such as risk of bias, indirectness, imprecision, inconsistency, and publication bias. Each outcome measure was categorized into one of four levels: "high," "moderate," "low," or "very low" quality. In the event of any discrepancies, they were resolved through discussion.

2.5.4. Data analysis

In this overview, a comprehensive analysis of acupuncture treatment for PSD was conducted, and summary estimates of relevant clinical effectiveness and outcomes were extracted from the included MAs. These summary estimates included measures, such as the odds ratio, risk ratio, weighted mean difference, standard mean difference, and 95% confidence intervals (CI). The results of AMSTAR-2 and PRISMA were summarized, with the data presented as percentages and frequencies for each item. Due to significant heterogeneity among the systematic reviews and considerable overlap of constituent RCTs, a quantitative synthesis was deemed infeasible.

3. Results

3.1. Study selection

A total of 2,593 articles were generated from the search across seven databases and were deemed eligible for screening. All studies were imported into EndNote for further screening. Following initial screening and removal of duplicates, 2,333 articles were excluded. After a full-text reading of 260 articles, a final selection of 13 articles

was included for review. Figure 1 illustrates the entire process of study selection. Based on the calculations, the overlap rate is 24.27% and the corrected covered area is 2.6%, indicating a slight overlap according to established thresholds (Table A1).[16,17]

3.2. Characteristics of the included MAs

All 13 MAs incorporated in this analysis exclusively comprised RCTs,[18-30] and the MAs were published between 2012 and 2023. Among these studies, there were eight publications in English[18-25] and five in Chinese,[26-30] with three of the Chinese publications originating from doctoral dissertations.[26-28] The included MAs conducted extensive searches across a spectrum of 11 – 29 databases, enlisting RCTs that ranged from 11 trials encompassing 1,225 participants[18] to 29 trials encompassing 2,394 participants.[29]

The diagnostic criteria for stroke incorporated into the review encompassed various standards, including the Diagnostic Points for Various Cerebrovascular Diseases, the Chinese Disease Diagnosis and Efficacy Standards, the *Chinese Classification of Cerebrovascular Diseases 2015*, and the *Chinese Guidelines for the Diagnosis and Treatment of Acute Ischemic Stroke 2018*, as well as confirmation through computed tomography or magnetic resonance imaging indicating the presence of stroke. In the context of depression, the diagnostic criteria included the *Chinese Classification and Diagnostics of Mental Disorders*,[31] the *Diagnostic and Statistical Manual of Mental Disorders*,[32,33] the Hamilton Depression Scale (HAMD),[34] and the *International Classification of Diseases*.

The intervention arms comprised acupuncture monotherapy, acupuncture adjunctive to pharmacological treatment, acupuncture combined with standard therapy, and acupuncture administered alongside a placebo. Various forms of acupuncture were employed, including electroacupuncture, manual acupuncture, scalp acupuncture, and the use of filiform needles. Control groups included modern medications, modern medications combined with sham acupuncture, or conventional therapy. Modern medications include fluoxetine, fluoxetine hydrochloride, sertraline, paroxetine, citalopram, venlafaxine, clomipramine, amitriptyline, alprazolam, triazolam, flupentixol, and melitracen, administered either individually or in combination.

Outcome measurements primarily consisted of four categories: Clinical effective rate, depression scores (HAMD scores and self-rating depression scale [SDS] scores), neurodeficiency scores (Barthel index, National Institutes of Health Stroke Scale [NIHSS] scores, neurofunctional deficit scores, and Chinese Stroke Clinical Neurological Functional Deficiency Severity Assessment), and safety outcomes (adverse events). Regarding methodological quality assessment, seven of the MAs opted for the Cochrane Risk of Bias tool to evaluate the methodological quality of the included RCTs,[18,20,21,26-28,30] while five MAs employed the Jadad scale or modified Jadad scale.[19,22,23,25,29] One MA concurrently reported both the Cochrane Risk of

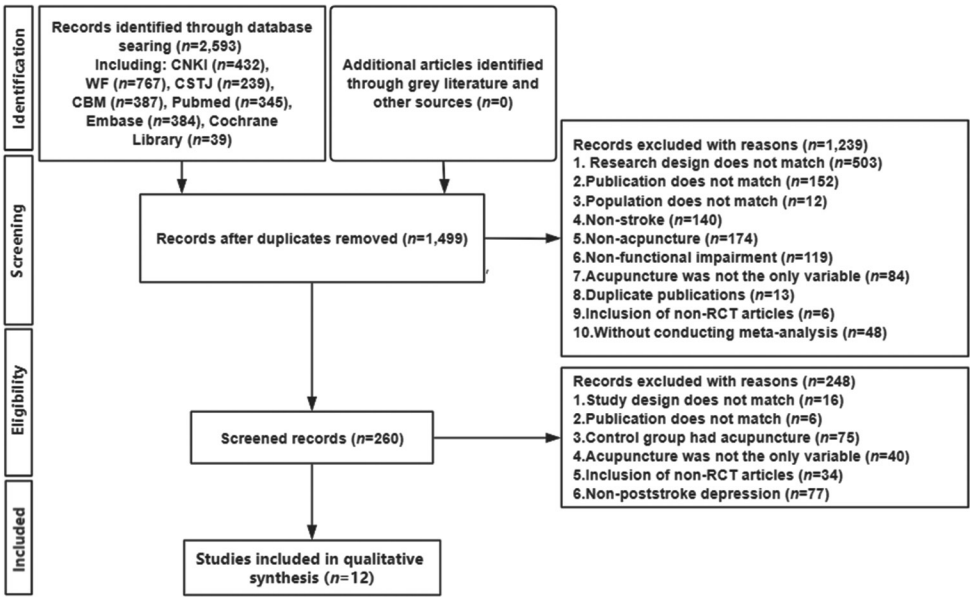


Figure 1. Flow diagram of the meta-analyses selection process
Abbreviations: CBM: Chinese Biomedical Literature Database; CNKI: China National Knowledge Infrastructure; CSTJ: China Science and Technology Journal Database; RCT: Randomized controlled trial; WF: Wanfang Database.

Bias tool and the modified Jadad assessment tool.[24] Safety assessments pertaining to acupuncture were conducted in 10 studies,[18,19,22-28,30] all of which concluded that acupuncture is safe. Finally, it is notable that only three MAs reported the funding sources (Table 1).[18,21,24]

3.3. Inclusion of MAs quality

3.3.1. Methodological quality

Assessments through the AMSTAR-2 tool classified the methodological quality of all 13 included MAs as very low (Table A2). Deficiencies were particularly noted in Items 2, 4, 7, 10, and 16 (Figure 2). Three MAs (15.4%) had registered their protocols before commencing their studies.[18,24,25] None of the MAs provided a list of excluded studies and demonstrated the rationale behind their exclusions. Only one MA (7.7%) disclosed the funding sources of its constituent RCTs,[18] while three MAs (23.1%) explicitly reported having no conflicts of interest.[18,21,24] Conversely, 11 MAs (84.6%) exhibited methodological shortcomings, including incomplete literature retrieval and insufficiently detailed reporting of RCT characteristics (Figure 3).

3.3.2. Report quality

The results of the PRISMA quality assessment are depicted in Figure 4. Among the 27 items assessed, 14 were reported as essentially complete, with a "yes" or "partly yes" response rate exceeding 90%. However, there were three items with inadequate reporting, with response rates of "yes" or "partly yes" falling below 50%.[35-37] The primary reporting deficiencies were observed in the following areas: Protocol and registration (3; 23.1%), search strategy (6; 46.2%), additional analyses (7; 53.8%), and funding (3; 23.1%).

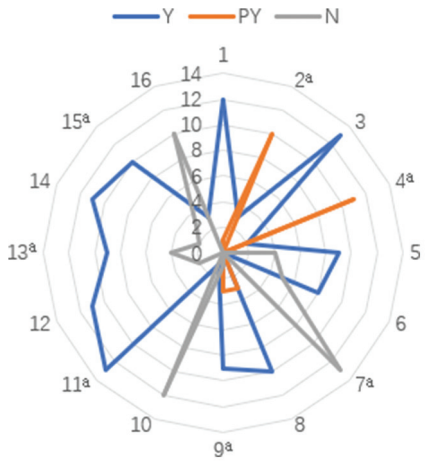


Figure 2. Graphical presentation of the results of A MeaSurement Tool to Assess Systematic Reviews 2 (AMSTAR-2)
Note: [a]Critical items of AMSTAR-2.
Abbreviations: N: No; PY: Partial yes; Y: Yes.

Detailed information regarding PRISMA can be found in Table A3.

3.3.3. Evidence quality

The evidence quality for the primary research outcomes extracted from the included MAs was assessed. In total, 61 study outcomes highlighted the effectiveness of acupuncture in the treatment of PSD. According to the GRADE assessment, among the 61 study outcomes, nine were of moderate quality, 29 were of low quality, and the remaining were of very low quality. Regarding limitations, all study outcomes were downgraded by one level. For

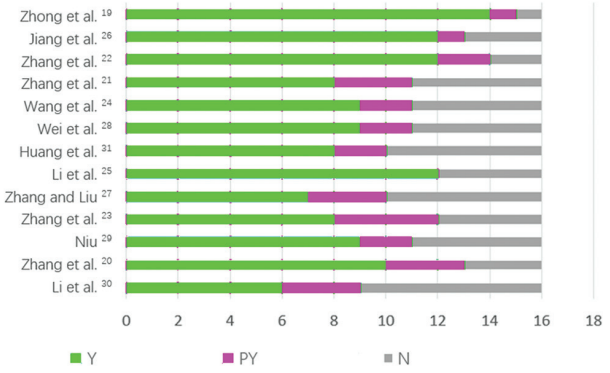


Figure 3. Graphical presentation of the results of a measurement tool to assess systematic reviews 2
Abbreviations: N: No; PY: Partial yes; Y: Yes.

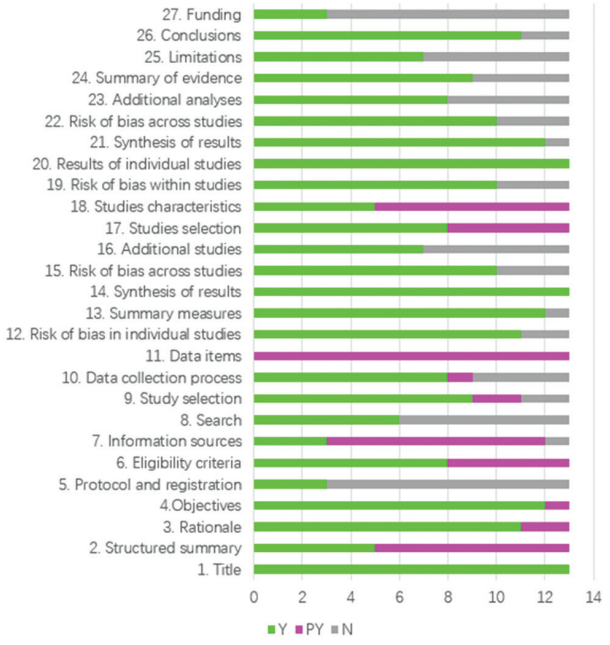


Figure 4. Graphical presentation of the results of Preferred Reporting Items for Systematic Reviews and Meta-analyses
Abbreviations: N: No; PY: Partial yes; Y: Yes.

Table 1. Characteristics of the included meta-analyses

Study	Databases	Diagnosis of stroke	Diagnosis of depression	Trials (subjects)	Intervention	Control	Quality assessment	Outcome	Safety	Conclusion
Li *et al.* (2012)[29]	Ovid Lww, PubMed, Cochrane Library, CNKI, CBM, WF, and CSTJ.	The Diagnostic Points for Various Cerebrovascular Diseases; the Chinese Disease Diagnosis and Efficacy Standards	CCMD; DSM; ICD	29 (2,394)	Acupuncture + modern medications	Modern medications	Jadad scale	①③	Unknown	The use of electroacupuncture may be effective in alleviating PSD, with its efficacy potentially not inferior to antidepressant medication. Electroacupuncture may also facilitate neurological recovery and improve the quality of life in stroke survivors
Zhang *et al.* (2014)[22]	Embase, Cochrane Library, PubMed, WOS, CNKI, CBM, and WF	Not specified	CCMD; DSM; HAMD	13 (845)	Acupuncture; Acupuncture + moxibustion; Electroacupuncture	Modern medications	Cochrane handbook	①⑤	Yes	Treating PSD with acupuncture is more effective compared with modern medication. The results are less reliable, and the quality of evidence is poor
Niu (2014)[28]	Embase, Cochrane Library, PubMed, CNKI, CBM, WF, and CSTJ	The Diagnostic Points for Various Cerebrovascular Diseases; the Chinese Disease Diagnosis and Efficacy Standards	CCMD; DSM; ICD	20 (1,372)	Electroacupuncture	Modern medications	Modified Jadad scale	①③⑤⑥⑧⑨⑪	Yes	The review of electroacupuncture treatment versus fluoxetine for PSD showed a significant difference in the total effective rates in HAMD and FMA scores, but failed to show a significant difference in the recovery of neurological function and daily activity
Zhang *et al.* (2014)[20]	Ovid Lww, PubMed, Cochrane Library, CNKI, CBM, WF, and CSTJ	The Diagnostic Points for Various Cerebrovascular Diseases; the Chinese Disease Diagnosis and Efficacy Standards	CCMD; DSM; ICD	17 (1,029)	Filiform needle	Modern medications	Modified Jadad scale	①②④	Yes	Early filiform needle acupuncture for PSD can effectively control depression. Filiform needle acupuncture is safe and reliable. The therapeutic effects of filiform needle acupuncture are better than those of antidepressant drugs

(Cont'd...)

Table 1. ***(Continued)***

Study	Databases	Diagnosis of stroke	Diagnosis of depression	Trials (subjects)	Intervention	Control	Quality assessment	Outcome	Safety	Conclusion
Zhang *et al.* (2017)[26]	Embase, CENTRAL, PubMed, CNKI, CBM, WF, and CSTJ	Not specified	Not specified	17 (1,522)	Electroacupuncture; Electroacupuncture + modern medications	Modern medications	Cochrane handbook	①③⑤⑦	Yes	The use of electroacupuncture may be effective in alleviating PSD, with its efficacy potentially not inferior to antidepressant medications. Electroacupuncture may also facilitate neurological recovery and improve the quality of life in stroke survivors. Importantly, electroacupuncture is considered safe
Li *et al.* (2018)[24]	Embase, CENTRAL, PubMed, WOS, CNKI, CBM, WF, and CSTJ	Confirmed by CT or MRI	CCMD; DSM; ICD	18 (813)	Electroacupuncture	Modern medications	Cochrane handbook; Jadad scale	①③⑤⑥⑧⑨	Yes	There is no significant difference between the effects of electroacupuncture and antidepressant medications on the severity of depression; however, electroacupuncture caused fewer adverse events
Huang *et al.* (2018)[30]	Embase, Cochrane Library, PubMed, Medline, CNKI, CBM, WF, and CSTJ	The Diagnostic Points for Various Cerebrovascular Diseases; confirmed by CT or MRI	CCMD	13 (1,193)	Electroacupuncture; Acupuncture; Acupuncture + modern medications	Modern medications	Cochrane handbook	①	Unknown	Acupuncture and electroacupuncture are effective therapeutic methods in improving the depressive state of patients with PSD. They can significantly improve the effects of treatment when combined with modern medications
Wei *et al.* (2020)[27]	Embase, Cochrane Library, PubMed, CNKI, CBM, and WF	The Diagnostic Points for Various Cerebrovascular Diseases; *Chinese Guidelines for the Diagnosis and Treatment of Acute Ischemic Stroke 2018*	CCMD; DSM; ICD	14 (1,640)	Acupuncture; Acupuncture + placebo	Modern medications; Modern medications+ sham acupuncture	Cochrane handbook	①②	Yes	Acupuncture intervention in patients with PSD is better than conventional modern medications in improving the HAMD score and curative effects. The number of adverse reactions to acupuncture in the treatment process is lower than that of common modern medications. The safety of acupuncture in the treatment of PSD patients is worth affirming to a certain extent

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Table 1. ***(Continued)***

Study	Databases	Diagnosis of stroke	Diagnosis of depression	Trials (subjects)	Intervention	Control	Quality assessment	Outcome	Safety	Conclusion
Wang *et al.* (2021)[21]	Embase, Cochrane Library, PubMed, CNKI, CBM, WF, and CSTJ	*Chinese Classification of Cerebrovascular Diseases 2015*; confirmed by CT or MRI	CCMD; HAMD	19 (1,606)	Acupuncture	Modern medications	Modified Jadad scale	①⑤	Yes	Compared with antidepressants, electroacupuncture is not less effective in improving depressive symptoms in PSD patients with greater safety
Zhang *et al.* (2021)[21]	Embase, Cochrane Library, Scopus, PubMed, Medline, CNKI, CBM, and WF	The Diagnostic Points for Various Cerebrovascular Diseases; confirmed by CT or MRI	CCMD; DSM	14 (1,124)	Acupuncture	Modern medications	Jadad scale	①②⑦⑧	Unknown	Acupuncture is superior to antidepressants in the treatment of PSD and reduces the incidence of adverse events. Therefore, acupuncture may be a meaningful treatment for PSD
Zhang *et al.* (2021)[21]	Embase, CENTRAL, PubMed, WOS, CNKI, WF, and CSTJ	Confirmed by CT or MRI	CCMD; DSM; ICD	13 (904)	Manual acupuncture + modern medications; Electroacupuncture + modern medications	Modern medications	Cochrane handbook	①②⑤⑥⑦	Unknown	Acupuncture combined with antidepressants shows a more favourable effect on the treatment of PSD than antidepressants alone
Jiang *et al.* (2023)[25]	Embase, Cochrane Library, PubMed, WOS, CNKI, CSTJ, CBM, and WF	Confirmed by CT or MRI	CCMD; DSM; ICD	14 (1,263)	Scalp acupuncture; Scalp acupuncture + electroacupuncture	Modern medications	Cochrane handbook	①②⑤⑩	Yes	Scalp acupuncture may confer more substantial improvements to depression and neurological function in PSD patients, with superior safety and definite clinical efficacy, compared to modern medication treatments.
Zhong *et al.* (2023)[18]	Embase, Cochrane Library, PubMed, WOS, CBM, and CNKI	Confirmed by CT or MRI	HAMD	11 (1,225)	Scalp acupuncture, Scalp acupuncture + conventional therapy	Conventional therapy	Cochrane handbook	①②③④⑤	Yes	While the effectiveness of scalp acupuncture combined with CT in treating PSD still requires further validation through rigorous randomized double-blind trials, this study provides a comprehensive collection of studies that meet the criteria for scalp acupuncture combined with CT in PSD treatment.

Notes: ①1Hamilton depression rating scale; ②Total clinical effective rate; ③Evaluation of self-rating depression scale (SDS); ④Safety evaluation; ⑤Adverse events; ⑥Barthel index score; ⑦National Institutes of Health Stroke Scale score; ⑧Treatment emergency symptom scale; ⑨Modified Edinburgh–Scandinavia stroke scale; ⑩Neurological deficit score; ⑪ Fugl–Meyer motor function assessment.
Abbreviations: CBM: Chinese Biomedical Literature Database; CCMD: *Chinese classification and diagnostic of mental disorders*; CENTRAL: Cochrane Central Register of Controlled Trials; CNKI: China National Knowledge Infrastructure; CSTJ: China Science and Technology Journal Database; CT: Computed tomography; DSM: *Diagnostic and statistical manual of mental disorders*; FMA: Fugl–Meyer motor function assessment; HAMD: Hamilton depression scale; ICD: *International classification of diseases*; MRI, Magnetic resonance imaging; PSD, Post-stroke depression; WF: Wanfang Database; WOS, Web of Science.

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inconsistency, 25 study outcomes were downgraded by one level due to inconsistency, while the remaining 36 study outcomes were not downgraded. Regarding imprecision, 38 study outcomes were downgraded by one level, and 23 were not downgraded. Regarding publication bias, 16 study outcomes were downgraded by one level, and 45 study outcomes were not downgraded. Indirect evidence indicated the highest level of evidence stability (Table 2).

3.4. Effectiveness and safety of acupuncture in the treatment of PSD

3.4.1. Clinical efficacy

A total of five MAs were included, focusing on clinical efficacy and cure rates.[18,20,21,25,27] Among them, two studies[20,25] provided moderate-quality evidence, while three studies[18,21,27] presented low-quality or very low-quality evidence. All five MAs reported that acupuncture, particularly methods, such as manual acupuncture, electroacupuncture, and scalp acupuncture, either used alone or in combination with modern medications or standard treatment, exhibited higher clinical efficacy compared to modern medications or standard treatment.[21,25] Nevertheless, one MA suggested no significant difference in improving PSD between electroacupuncture in conjunction with modern medications and modern medications alone.[21] In addition, one study[27] suggested that acupuncture treatment for PSD had a higher clinical cure rate compared to modern medications.

3.4.2. Depression scores

Six MAs[20,22,23,25-28] reported that acupuncture treatment, especially scalp acupuncture, electroacupuncture, and filiform needle, could improve HAMD scores in PSD patients compared to standalone modern medications. The quality of evidence varied, with some studies rated as moderate,[25,28] low,[20,22,23,25,26] or very low.[20,22] However, three MAs[23,24,30] reported no significant difference in improving HAMD scores between acupuncture and modern medication treatments. In addition, one study[25] found that modern medications were superior to acupuncture in improving HAMD scores.

Five MAs[18,21,24,26,30] reported that acupuncture combined with modern medications or standard treatment could improve HAMD scores in PSD patients compared to standalone modern medications or standard treatment. However, three MAs[18,21,30] reported that scalp acupuncture, manual acupuncture, and electroacupuncture combined with modern medications or standard treatment did not significantly differ from modern medications or standard treatment alone in improving HAMD scores.

Regarding one of the primary outcomes, the SDS, one study[18] concluded that scalp acupuncture combined with conventional treatment was superior to conventional treatment alone in improving SDS scores in PSD patients, while another study[26] reported no significant difference between acupuncture and modern medications in improving SDS scores.

3.4.3. Neurological function scores

One MA[21] demonstrated that acupuncture produced significantly greater improvements in the Barthel index among PSD patients than pharmacotherapy alone. In contrast, two MAs[26,28] reported no significant differences between either acupuncture monotherapy or acupuncture combined with pharmacotherapy versus pharmacotherapy alone. However, one of the MAs[28] reported that acupuncture had a better effect in improving the Fugl–Meyer assessment of motor function and modified Edinburgh–Scandinavian Stroke Scale scores in PSD patients compared to standalone modern medication treatment. Meanwhile, two MAs[25,26] suggested that acupuncture or acupuncture combined with modern medications could improve the neurological deficit score in PSD patients compared to standalone modern medications. Two MAs[20,21] suggested that acupuncture or acupuncture combined with modern medications could improve the NIHSS score in PSD patients compared to standalone modern medications. One MA demonstrated that acupuncture was superior to modern medications in improving the treatment of the emergency symptom scale score in PSD patients.[20]

3.4.4. Adverse events

Four MAs[19,23-25] assessed adverse events, and all indicated that acupuncture had better safety compared to modern medical treatment.

4. Discussion

This overview synthesized and critically appraised existing MAs of acupuncture for PSD to evaluate its therapeutic efficacy and safety. The methodological rigor, risk of bias, reporting completeness, and outcome data of each MA were assessed to gauge the reliability of the present evidence and inform future research directions. Nevertheless, persistent inconsistencies across studies and the generally low quality of available data preclude definitive conclusions regarding the benefits of acupuncture for PSD.

4.1. Summary of key findings

Based on the inclusion criteria and following comprehensive searching and deduplication, a total of 13 MAs on the efficacy and safety of acupuncture therapy for PSD were ultimately selected. Notably, the quality of the included studies demonstrated a gradual improvement over the years, as evidenced by increasing adherence to the criteria of various evaluation tools.

Table 2. Results of the GRADE assessment

Reference	Interventions	Outcomes	Studies (participants)	Effect (95% CI)	Limitations	Inconsistency	Indirectness	Imprecision	Publication bias	Quality
Wei *et al.* (2020)[27]	Acupuncture versus modern medications	Cure rate	9 (655/657)	RR 1.48 (1.23 – 1.78)	−1①	0	0	0	−1④	Low
Wei *et al.* (2020)[27]	Acupuncture versus modern medications	Effective rate	11 (709/708)	RR 1.13 (1.08 – 1.17)	−1①	0	0	0	−1④	Low
Zhang *et al.*[19]	Manual acupuncture + modern medications versus modern medications	Effective rate	4 (139/137)	RR 1.38 (1.20 – 1.58)	−1①	0	0	−2③	0	Very low
Zhang *et al.*[19]	Electroacupuncture + modern medications versus modern medications	Effective rate	2 (47/46)	RR 1.21 (0.99 – 1.48)	−1①	0	0	−2③	0	Very low
Zhang *et al.*[20]	Manual acupuncture + modern medications versus modern medications; Electroacupuncture + modern medications versus modern medications	Effective rate	6 (186/183)	RR 1.33 (1.19 – 1.49)	−1①	0	0	−1③	0	Low
Zhang *et al.* (2021)[21]	Acupuncture versus modern medications	Effective rate	12 (507/503)	MD 1.15 (1.08 – 1.21)	−1①	0	0	0	0	Moderate
Jiang *et al.* (2023)[25]	Scalp acupuncture versus modern medications; Scalp acupuncture + electroacupuncture versus modern medications	Effective rate	12 (553/543)	RR 1.09 (1.02 – 1.16)	−1①	0	0	0	0	Moderate
Zhong *et al.* (2023)[18]	Scalp acupuncture + conventional treatment versus conventional treatment	Effective rate	8 (328/323)	OR 2.44 (1.61 – 3.70)	−1①	0	0	0	−1④	Low
Li *et al.* (2012)[29]	Acupuncture + modern medications versus modern medications	HAMD score	8 (266/265)	MD−2.20 (−2.62 – −1.79)	−1①	0	0	0	−2④	Very low
Zhang *et al.* (2014)[22]	Acupuncture versus modern medications; Acupuncture + moxibustion versus modern medications; Electroacupuncture versus modern medications	HAMD score	12 (347/417)	MD 0.26 (0.11 – 0.40)	−2①	−1②	0	0	0	Very low

(Cont'd...)

Table 2. ***(Continued)***

Reference	Interventions	Outcomes	Studies (participants)	Effect (95% CI)	Limitations	Inconsistency	Indirectness	Imprecision	Publication bias	Quality
Zhang *et al.* (2017)[26]	Electroacupuncture versus modern medications	HAMD score	9 (434/432)	MD−0.50 (−0.92 – −0.08)	−1①	−1②	0	0	0	Low
Zhang *et al.* (2017)[26]	Electroacupuncture + modern medications versus modern medications	HAMD score	9 (298/293)	WMD−2.50 (−2.93 – −2.06)	−1①	0	0	−1③	0	Low
Li *et al.* (2018)[24]	Electroacupuncture versus modern medications	HAMD score	15 (636/595)	SMD−0.04 (−0.18 – 0.10)	−1①	0	0	0	0	Moderate
Huang *et al.* (2018)[30]	Acupuncture versus modern medications	HAMD score	3 (152/196)	MD−0.54 (−1.46 – 0.39)	−1①	0	0	−1③	−1○44	Very low
Huang *et al.* (2018)[30]	Electroacupuncture versus modern medications	HAMD score	5 (200/197)	MD 0.13 (−1.07 – 1.33)	−1①	−1②	0	−1③	−1○44	Very low
Huang *et al.* (2018)[30]	Acupuncture + modern medications versus modern medications	HAMD score	3 (98/96)	MD−3.28 (−4.45 – −2.12)	−1①	0	0	0	−1○44	Low
Huang *et al.* (2018)[30]	Electroacupuncture + modern medications versus modern medications	HAMD score	2 (79/80)	MD−0.39 (−8.29 – 2.74)	−1①	−1②	0	0	−1○44	Very low
Wei *et al.* (2020)[27]	Acupuncture versus modern medications; Acupuncture + placebo versus modern medications + sham needle	HAMD score	14 (814/813)	MD−3.87 (−5.22–2.52)	−1①	−1②	0	0	−1○44	Very low
Zhang *et al.* (2021)[19]	Manual acupuncture + modern medications versus modern medications	HAMD score	6 (244/239)	MD−3.54 (−4.54 – 2.55)	−1①	−1②	0	−1③	0	Very low
Zhang *et al.* (2021)[19]	Electroacupuncture + modern medications versus modern medications	HAMD score	7 (212/209)	MD−3.66 (−4.58–−2.74)	−1①	0	0	−1③	0	Low
Zhang *et al.* (2021)[19]	Manual acupuncture + modern medications versus modern medications; Electroacupuncture + modern medications versus modern medications	HAMD score	13 (456/448)	MD−3.60 (−4.25 – −2.95)	−1①	0	0	0	0	Moderate

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Table 2. ***(Continued)***

Reference	Interventions	Outcomes	Studies (participants)	Effect (95% CI)	Limitations	Inconsistency	Indirectness	Imprecision	Publication bias	Quality
Jiang *et al.* (2023)[25]	Scalp acupuncture versus modern medications	HAMD score	8 (290/283)	MD−2.75 (−4.84 – −0.65)	−1①	−1②	0	0	0	Low
Jiang *et al.* (2023)[25]	Scalp acupuncture + electroacupuncture versus modern medications	HAMD score	2 (61/60)	MD−1.07 (−1.60 – −0.55)	−1①	0	0	0	0	Moderate
Zhong *et al.* (2023)[18]	Scalp acupuncture + conventional treatment versus conventional treatment	HAMD score	7 (427/407)	MD−3.79 (−8.51 – 0.92)	−1①	−1②	0	0	−1④	Very low
Zhang *et al.* (2014)[20]	Acupuncture versus modern medications	HAMD score (2 weeks after treatment)	4 (98/94)	MD−2.34 (−3.46 – −1.22)	−1①	0	0	−2③	0	Very low
Zhang Lincheng, (2021)[18]	Acupuncture versus modern medications	HAMD score (3 weeks after treatment)	2 (70/70)	MD−1.17 (−2.18 – −0.16)	−1①	0	0	−2③	0	Very low
Zhang *et al.* (2014)[22]	Acupuncture versus modern medications	HAMD score (4 weeks after treatment)	7 (191/191)	MD−0.49 (−1.72 – −0.74)	−1①	−1②	0	−1③	0	Very low
Li *et al.* (2018)[24]	Electroacupuncture versus modern medications	HAMD score (4 weeks after treatment)	7 (272/231)	SMD−0.11 (−0.31 – 0.10)	−1①	0	0	−1③	0	Low
Wang *et al.* (2021)[23]	Acupuncture versus modern medications	HAMD score (4 weeks after treatment)	13 (468/426)	SMD−0.30 (−0.58 – −0.01)	−1①	−1②	0	0	0	Low
Zhang *et al.* (2021)[21]	Acupuncture versus modern medications	HAMD score (4 weeks after treatment)	3 (91/84)	MD−4.44 (−5.64 – −3.23)	−1①	−1②	0	−2③	0	Very low
Li *et al.* (2018)[24]	Electroacupuncture versus modern medications	HAMD score (6 weeks after treatment)	3 (93/93)	SMD 0.04 (−0.43 – 0.51)	−1①	−1②	0	−2③	0	Very low
Wang *et al.* (2021)[23]	Acupuncture versus modern medications	HAMD score (6 weeks after treatment)	4 (133/133)	SMD 0.04 (−0.38 – 0.36)	−1①	0	0	−1③	0	Low
Zhang *et al.* (2021)[21]	Acupuncture versus modern medications	HAMD score (6 weeks after treatment)	6 (205/201)	MD−1.20 (−1.92 – −0.49)	−1①	−1②	0	−1③	0	Very low
Li *et al.* (2018)[24]	Electroacupuncture versus modern medications	HAMD score (8 weeks after treatment)	5 (271/271)	SMD−0.01 (−0.23 – 0.22)	−1①	0	0	−1③	0	Low
Wang *et al.* (2021)[23]	Acupuncture versus modern medications	HAMD score (8 weeks after treatment)	5 (271/271)	SMD−0.01 (−0.23 – 0.22)	−1①	0	0	0	0	Moderate
Zhang *et al.* (2021)[21]	Acupuncture versus modern medications	HAMD score (8 weeks after treatment)	4 (224/224)	MD−4.33 (−4.96 – −3.70)	−1①	0	0	−1③	0	Low
Niu (2014)[28]	Electroacupuncture versus modern medications	The reduction rate of the HAMD score	11 (496/490)	OR 1.61 (1.09 – 2.38)	−1①	0	0	0	0	Moderate

(Cont'd...)

Table 2. *(Continued)*

Reference	Interventions	Outcomes	Studies (participants)	Effect (95% CI)	Limitations	Inconsistency	Indirectness	Imprecision	Publication bias	Quality
Zhang *et al.* (2014)[20]	Filiform needle versus modern medications	The reduction rate of the HAMD score (4 weeks after treatment)	8 (211/211)	RR 1.11 (1.03 – 1.21)	−1①	0	0	−1③	0	Low
Zhang *et al.* (2014)[20]	Acupuncture versus modern medications	The reduction rate of the HAMD score (6 weeks after treatment)	3 (93/90)	RR 1.10 (0.98 – 1.28)	−1①	0	0	−2③	0	Very low
Zhang *et al.* (2017)[26]	Electroacupuncture versus modern medications	SDS score	2 (96/93)	MD−1.91 (−9.51 – 5.70)	−1①	−1②	0	−2③	0	Very low
Zhong *et al.* (2023)[18]	Scalp acupuncture + conventional treatment versus conventional treatment	SDS score	3 (139/135)	MD−8.72 (−9.71 – −7.73)	−1①	0	0	0	−1④	Low
Niu (2014)[28]	Electroacupuncture versus modern medications	Barthel index	5 (171/171)	WMD 1.38 (−1.88 – 4.65)	−1①	−1②	0	−1③	−1④	Very low
Zhang *et al.* (2017)[26]	Electroacupuncture+ modern medications versus modern medications	Barthel index	2 (85/82)	WMD−11.59 (−55.86 – 32.67)	−1①	−1②	0	−2③	0	Very low
Zhang *et al.* (2021)[19]	Manual acupuncture + modern medications versus modern medications	Barthel index	2 (105/102)	MD 6.11 (2.40 – 9.82)	−1①	0	0	−1③	0	Low
Zhang *et al.* (2021)[19]	Electroacupuncture + modern medications versus modern medications	Barthel index	1 (40/40)	MD 10.93 (6.50 – 15.36)	−1①	−1②	0	−2③	0	Very low
Zhang *et al.* (2021)[19]	Manual acupuncture + modern medications versus modern medications; Electroacupuncture + modern medications versus modern medications	Barthel index	3 (145/142)	MD 8.10 (5.25 – 10.94)	−1①	0	0	−1③	0	Low
Niu (2014)[28]	Electroacupuncture versus modern medications	Fugl–Meyer motor function assessment	2 (76/76)	WMD 3.50 (0.14 – 6.86)	−1①	−1②	0	−2③	−1④	Very low
Niu (2014)[28]	Electroacupuncture versus modern medications	MESS score	2 (65/65)	WMD−1.89 (−4.77 – 0.99)	−1①	−1②	0	−2③	−1④	Very low
Niu (2014)[28]	Electroacupuncture versus modern medications	The reduction rate of the MESS score	2 (65/65)	OR 1.80 (0.61 – 5.31)	−1①	0	0	−2③	−1④	Very low

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Table 2. ***(Continued)***

Reference	Interventions	Outcomes	Studies (participants)	Effect (95% CI)	Limitations	Inconsistency	Indirectness	Imprecision	Publication bias	Quality
Zhang *et al.* (2017)[26]	Electroacupuncture versus modern medications	Neurological deficit score	2 (67/67)	MD−3.72 (−5.51 – −1.94)	−1①	0	0	−2③	0	Very low
Zhang *et al.* (2017)[26]	Electroacupuncture + modern medications versus modern medications	Neurological deficit score	2 (52/50)	WMD−6.31 (−9.28 – −3.34)	−1①	−1②	0	−2③	0	Very low
Jiang *et al.* (2023)[25]	Scalp acupuncture versus modern medications; Scalp acupuncture + electroacupuncture versus modern medications	Neurological deficit score	6 (196/190)	MD−3.06 (−5.91 – −0.21)	−1①	−1②	0	−1③	0	Very low
Zhang *et al.* (2021)[19]	Manual acupuncture + modern medications versus modern medications	NIHSS score	3 (154/149)	MD−2.29 (−3.69 – −0.90)	−1①	−1②	0	−1③	0	Very low
Zhang *et al.* (2021)[19]	Electroacupuncture + modern medications versus modern medications	NIHSS score	1 (43/43)	MD−2.70 (−3.72 – −1.68)	−1①	−1②	0	−2③	0	Very low
Zhang *et al.* (2021)[19]	Manual acupuncture + modern medications; Electroacupuncture + modern medications versus modern medications	NIHSS score	4 (197/192)	MD−2.39 (−3.37 – −1.41)	−1①	−1②	0	−1③	0	Very low
Zhang *et al.* (2021)[18]	Acupuncture versus modern medications	NIHSS score	2 (154/154)	MD−2.31 (−2.53 – −2.09)	−1①	0	0	−1③	0	Low
Zhang *et al.* (2021)[21]	Acupuncture versus modern medications	TESS score	3 (162/161)	MD−4.70 (−4.93 – −4.48)	−1①	−1②	0	−1③	0	Very low
Zhang *et al.* (2014)[22]	Acupuncture versus modern medications; Acupuncture + moxibustion versus modern medications; Electroacupuncture versus modern medications	Adverse events	8 (251/251)	RR 0.32 (0.19 – 0.53)	−2①	0	0	−1③	0	Very low
Li *et al.* (2018)[24]	Electroacupuncture versus modern medications	Adverse events	8 (397/401)	RR 0.21 (0.14 – 0.33)	−1①	0	0	0	0	Moderate

(Cont'd...)

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Table 2. ***(Continued)***

Reference	Interventions	Outcomes	Studies (participants)	Effect (95% CI)	Limitations	Inconsistency	Indirectness	Imprecision	Publication bias	Quality
Wang *et al.* (2021)[23]	Acupuncture versus modern medications	Adverse events	9 (428/431)	RR 0.21 (0.14 – 0.32)	−1①	0	0	0	0	Moderate
Jiang *et al.* (2023)[25]	Scalp acupuncture versus modern medications; Scalp acupuncture + electroacupuncture versus modern medications	Adverse events	5 (270/266)	RR 0.12 (0.05 – 0.29)	−1①	0	0	0	0	Moderate

Notes: −2: Downgrade by two levels; −1: Downgrade by one level; 0: Not downgrade; ① The experimental designs of included studies exhibit significant bias in terms of randomization, blinding, allocation concealment, data integrity, or selective reporting; ② There is limited overlap in confidence intervals, a small *p*-value in the heterogeneity test, or a high I^2 value; ③The sample size is small, or the confidence intervals are wide; ④ The funnel plot is asymmetric, or there is a limited number of studies included.

Abbreviation: GRADE: Grading of recommendations, assessment, development, and evaluation; RR: Risk ratio; OR: Odds ratio; MD: Weighted mean difference; SMD: Standardized mean difference; WMD: Weighted mean difference; HAMD: Hamilton rating scale for depression; SDS: Self-rating depression scale; MESS: Modified Edinburgh–Scandinavia stroke scale; NIHSS: National Institutes of Health Stroke Scale; TESS: Treatment emergency symptom scale.

According to AMSTAR-2 criteria, the quality of all included MAs was rated as very low. Primary methodological deficiencies include failing to publish pre-registered study protocols, lacking comprehensive literature search strategies, lacking detailed exclusion criteria lists, non-disclosure of funding sources for included RCTs, and failing to report conflicts of interest. Among the critical items, substantial reporting deficiencies were observed in Item 2 (23.08%), Item 4 (15.38%), and Item 7 (0%). The main reasons are as follows: (i) Only three MAs[18,24,25] registered a protocol in advance and provided a registration number; (ii) only two MAs[18,24] employed a comprehensive and appropriate literature search strategy; and (iii) None of the included MAs explicitly listed the excluded studies or provided reasons for each exclusion.

Results from the PRISMA checklist indicated that none of the MAs completed all 27 items, highlighting five major deficiencies that warrant attention: (i) The absence of reporting study protocols and registration, (ii) incomplete reporting of database search strategies, (iii) absence of additional analyses, (iv) limitations of the studies, and (v) reporting of funding support.

In the GRADE evaluation, "risk of bias" was the primary factor leading to downgrading, followed by "imprecision." Methodological shortcomings in the included trials – such as inadequate randomization, lack of blinding, insufficient allocation concealment, incomplete data, and selective reporting – resulted in every outcome being penalized for study bias. Furthermore, small sample sizes and wide CIs also introduced imprecision into the findings.

4.2. Comparison with previous studies

Prior research has predominantly focused on the efficacy and safety of acupuncture for post-stroke neurological deficits,[38-40] pain,[41] and cognitive impairment,[42] with relatively fewer systematic investigations targeting PSD. Publications have also reported using acupuncture to treat depression,[43] depression comorbid with cardiovascular disease,[44] cancer-related depression,[45] post-trigeminal neuralgia depression,[46] perimenopausal depression,[47] and postpartum depression.[48,49]

Notably, there are presently three overviews of systematic reviews on similar topics (two in Chinese and one in English), all conducted at least five years ago.[50-52] One such study, which included 12 systematic reviews, evaluated non-invasive acupuncture interventions – such as auricular acupuncture and acupoint plasters – with comparators, including traditional Chinese herbs, proprietary Chinese medicines, and psychotherapy. Another overview, incorporating 10 systematic reviews, reported findings for two primary outcomes – HAMD

scores and adverse event rates – that were directionally consistent with the present results.[52]

Building on these foundations, the present study incorporated several recently published, high-quality RCTs, thereby expanding both the sample size and the breadth of included research. In addition to using the HAMD as the primary outcome measure, the SDS was also included to capture subjective depressive symptoms, as well as incorporating various functional and quality-of-life scales that indirectly reflect the impact of depression and its comorbid features. Moreover, the effects of different needling techniques and treatment regimens were systematically catalogued, and subgroup analyses were conducted. Overall, the findings align with previous reviews in supporting acupuncture's efficacy for PSD, although some individual trials did not demonstrate significant differences. By adopting stricter inclusion criteria, a more comprehensive search strategy, and a more rigorous quality-assessment framework, this study has further minimized heterogeneity and strengthened the interpretability of the evidence. In particular, the detailed analysis of "acupuncture modalities" and "acupuncture combined with medications" addresses gaps in earlier literature.

Traditional Chinese medicine emphasizes syndrome differentiation, leading to individualized acupuncture protocols in which acupoint selection, needle retention time, treatment frequency, and overall course are tailored to each patient's specific presentation. Previous research has identified GV20 as the most frequently used acupoint for treating depression, followed by GV24+, LR3, LI4, PC6, and HT7.[53] Given the heterogeneity of clinical manifestations during depressive episodes, core acupoints are often supplemented with additional, personalized selections. For example, liver qi stagnation is typically addressed with a combination of BL18, LR14, LI4, LR3, and RN17. When stagnated qi transforms into fire, acupoints, such as LR2, ST44, SJ6, and GB43 are employed. Patterns characterized by grief and mental distress frequently involve PC6, HT5, and BL15, whereas cases of heart–spleen deficiency tend to use BL15, BL20, and ST36. For yang deficiency, RN5 and RN4 are recommended.[54] Although specific acupoint combinations vary slightly between studies, all adhere rigorously to the diagnostic and therapeutic principles of acupuncture.

Moreover, needle manipulation techniques are critical for eliciting *deqi*, the characteristic "arrival of qi" sensation. One study reported that high-frequency rotating manipulations at scalp acupoints can transmit needle sensations to the pathological focus, thereby regulating the primordial spirit and enhancing therapeutic effects. After rapid needle insertion, applying small unidirectional rotations at limb acupoints prolongs the *deqi* sensation, further augmenting efficacy. In addition, prior experimental data have confirmed that prolonged needle retention can significantly improve somatic symptoms and sleep disturbances – a benefit that is particularly evident in patients with mild depression.[55]

By delving into the mechanisms of PSD, some promising techniques have the potential to lay the foundation for early diagnosis and quantitative assessment of PSD.[56] Research indicates that the location of post-stroke damage is a crucial factor in depression, with individuals having lesions near the frontal lobe and basal ganglia in the left hemisphere more prone to depression and cognitive deficits.[57,58] Computed tomography has revealed an enlarged ratio of lateral ventricles and the third ventricle to brain volume in patients with PSD. In addition, lacunar infarctions resulting from occlusions in deep hemispheric regions, a consequence of intracranial atherosclerosis, are more likely to lead to depression following a stroke.[59] Furthermore, functional imaging studies have identified specific alterations in the functional connectivity between PSD and the dorsolateral pre-frontal cortex within the depression circuit.[60-62] These impactful changes are strongly associated with the severity of depression in PSD and may serve as optimal targets for repetitive transcranial magnetic stimulation therapy. Moreover, significant correlations are observed between stroke progression-related quantitative electroencephalography parameters, such as delta/alpha ratio or delta/theta/alpha ratio (DTABR), and hospital anxiety and depression scale-depression subscale (HADS-D) scores. Elevated DTABR can serve as early indicators of neuropsychiatric disorders before changes in depressive symptoms and HADS-D scores become evident, and these have been applied in the context of neurological and psychiatric symptom monitoring.[63,64] DTABR can also be employed to assess the dominance of slow or fast waves in patients with ischemic stroke, which may be associated with increased depressive symptoms and depressive tendencies.[65,66] Exploring the disease mechanisms contributes to the identification of measurable indicators for PSD, providing a reference for the assessment of subsequent clinical treatments.

4.3. Implications for future research

This overview has identified methodological, reporting, and evidence quality deficiencies in the included literature, resulting in a relatively weak level of evidence for the studies. To enhance the quality of methodology, future research should draft study protocols and register them on platforms, such as Cochrane before conducting MAs. This practice contributes to maximizing research transparency and reducing the risk of methodological bias. Second,

literature searches should be as comprehensive as possible, including references from studies and grey literature searches, which are particularly important. Furthermore, studies should provide more detailed information about exclusion lists and reasons to ensure research replicability. Publication bias should be examined when a sufficient number of studies are included. When heterogeneity in the results of data synthesis analysis is significant, efforts should be made to identify the sources of heterogeneity through subgroup analyses or meta-regression, where possible, and explain the impact of bias risk in the discussion. In addition, MAs should comprehensively describe the characteristics of the RCTs they include, aiding in clarifying the sources of heterogeneity. Reporting funding sources for both the MA and individual RCTs is essential to identify potential conflicts of interest. Finally, two or more researchers should independently and repeatedly conduct screening and data extraction. Following these methods and reporting standards will ensure the quality of MAs.

According to the GRADE approach for evaluating the quality of evidence, a total of seven moderate-quality studies were identified in this review. Specifically, two moderate-quality studies suggest that acupuncture, scalp acupuncture, and scalp acupuncture combined with electroacupuncture are more effective than conventional modern medications alone in improving the overall treatment efficacy for PSD. Another two moderate-quality studies indicate that acupuncture shows no significant difference compared to modern medications alone in improving HAMD scores. One moderate-quality study demonstrates that electroacupuncture is superior to modern medications alone in reducing HAMD scores in PSD patients. In addition, two moderate-quality studies support that acupuncture combined with modern medications is more effective than modern medications alone in improving depressive symptoms. Furthermore, three moderate-quality studies suggest that acupuncture, electroacupuncture, scalp acupuncture, and scalp acupuncture combined with electroacupuncture are associated with a lower incidence of adverse events compared to modern medications alone. The remaining evidence in this review was rated as low or very low quality. Future research should focus on standardizing methods, such as randomization, blinding, and allocation concealment in the trial design process, striving to provide high-quality, large-sample, RCT evidence.

This study suggests that acupuncture treatment may help improve HAMD scores and enhance the overall clinical efficacy in patients with PSD, with a lower risk of adverse events compared to conventional pharmacological therapy. However, substantial heterogeneity was observed among the included systematic reviews, which may affect the stability and interpretability of the results. The potential sources of heterogeneity include:

(i). Variability in patient characteristics: Some reviews did not clearly stratify patients by stroke type, disease stage, or depression severity, leading to heterogeneity in the study populations.

(ii). Inconsistent intervention duration: There is currently no standardized treatment course for acupuncture in PSD, and some reviews did not specify the duration of intervention, resulting in limited comparability of treatment exposure.

(iii). Diverse outcome assessment tools: A wide range of depression-related scales were used across the included reviews, lacking uniformity, which compromises the consistency and reliability of outcome evaluation.

These heterogeneity factors may confound the overall judgment of acupuncture efficacy. Future studies should improve methodological rigor, ensure clear stratification of patient populations, standardize intervention protocols, and adopt consistent outcome measures to enhance the quality of evidence and clinical applicability.

4.4. Limitations

This study inevitably has some limitations. First, the overall assessment's robustness is directly limited by the poor quality of the original studies. In addition, describing and assessing acupuncture's point selection and operational techniques was impossible because most of the included SRs did not specify these parameters. Finally, the subjectivity of assessors may lead to bias; hence, the reported results must be interpreted cautiously.

5. Conclusion

Based on the evidence provided in this overview, acupuncture appears to be a safe therapeutic approach and may potentially serve as an effective complementary treatment for PSD to some extent. However, due to the low quality of evidence from the included studies and inconsistent conclusions, this conclusion should be approached with caution. It is recommended that future reviews adhere to recognized reporting standards to enhance the quality of evidence.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

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Writing – review & editing: All authors

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

The datasets analyzed during the present study are available from the corresponding author on reasonable request.

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Appendices

Table A1. Summary of duplicate studies

Serial number	Duplicate article title	Frequency of duplication
1	Effects of electric-acupuncture and fluoxetine on depression and neurological function of post-stroke depression patients	3
2	Clinical observation on the efficacy of electroacupuncture in the treatment of post-stroke depression (in Chinese)	3
3	Interventional effects of point-through-point acupuncture on post-stroke depression and its influence on plasma cortisol levels (in Chinese)	2
4	Clinical observation on treatment of post-stroke depression with back-shu point-based acupuncture (in Chinese)	2
5	Clinical study on acupuncture combined with pharmacotherapy for post-stroke depression (in Chinese)	2
6	Clinical observation of electroacupuncture in the treatment of post-stroke depression (Chen, 2012)[14]	2
7	Clinical observation of electroacupuncture in the treatment of post-stroke depression (Long *et al*., 2004)[15]	2
8	Clinical observation of electroacupuncture with different current frequencies for post-stroke depression	2
9	Clinical observation of electroacupuncture for elderly post-stroke depression	2
10	Observation on the effect of "*Xingshen Jieyu*" acupuncture method in treating post-stroke depression	2
11	A clinical observation of acupuncture treatment of 72 cases with post-stroke depression	2
12	Clinical research into the treatment of PSD with an electrical needle for invigorating the brain and easing mental stress	2
13	Effect of electroacupuncture on regional cerebral blood flow in patients with post-stroke depression	2
14	Electroacupuncture versus fluoxetine capsule for post-stroke depression: a randomized controlled trial	2
15	Clinical observation on head point-through-point electroacupuncture for treatment of post-stroke depression	2
16	Clinical observation on combined acupuncture and pharmacotherapy in the treatment of 30 cases of post-stroke depression (in Chinese)	2
17	Clinical observation on electroacupuncture combined with fluoxetine (Prozac) for post-stroke depression (in Chinese)	2
18	Clinical study on electroacupuncture combined with fluoxetine for post-stroke depression in young patients (in Chinese)	2
19	Observation on oral fluoxetine combined with acupuncture at eight confluent points in the treatment of post-stroke depression (in Chinese)	2
20	Effect of electroacupuncture combined with fluoxetine on depressive status and neurological function in patients with post-stroke depression (in Chinese)	2
21	Clinical observation on scalp electroacupuncture combined with "*Kai Siguan*" method for post-stroke depression (in Chinese)	2
22	Clinical observation on electroacupuncture combined with paroxetine for post-stroke depression (in Chinese)	2
23	Randomized controlled trial of electroacupuncture and fluoxetine capsule in the treatment of post-stroke depression (in Chinese)	2
24	Clinical observation of head point-through-point electroacupuncture for post-stroke depression (in Chinese)	2
		50

Table A2. Results of the AMSTAR-2 assessment

Reference	1	2[a]	3	4[a]	5	6	7[a]	8	9[a]	10	11[a]	12	13[a]	14	15[a]	16	Compliance (*n* [%])	Overall quality
Li *et al.* (2012)[1]	Y	PY	Y	PY	N	N	N	PY	Y	N	Y	Y	Y	N	N	N	6 (37.50)	Very low
Zhang *et al.* (2014)[2]	Y	PY	Y	PY	Y	Y	N	Y	PY	N	Y	Y	Y	Y	Y	N	10 (62.50)	Very low
Niu. (2014)[3]	Y	PY	Y	PY	Y	Y	N	Y	Y	N	Y	N	N	Y	Y	N	9 (56.25)	Very low
Zhang *et al.* (2014)[4]	Y	PY	Y	PY	N	Y	N	PY	PY	N	Y	Y	Y	Y	Y	N	8 (50.00)	Very low
Zhang and Liu (2017)[5]	Y	PY	Y	PY	Y	Y	N	PY	Y	N	Y	N	N	N	Y	N	7 (43.75)	Very low
Li *et al.* (2018)[6]	Y	Y	Y	Y	N	N	N	Y	Y	N	Y	Y	Y	Y	Y	Y	12 (75.00)	Very low
Huang *et al.* (2018)[7]	Y	PY	Y	PY	Y	N	N	Y	Y	N	Y	Y	N	Y	N	N	8 (50.00)	Very low
Wei *et al.* (2020)[8]	Y	PY	Y	PY	Y	N	N	Y	Y	N	Y	Y	Y	Y	N	N	9 (56.25)	Very low
Wang *et al.* (2021)[9]	Y	PY	Y	PY	Y	N	N	Y	N	N	Y	Y	Y	Y	Y	N	9 (56.25)	Very low
Zhang *et al.* (2021)[10]	PY	PY	Y	PY	N	Y	N	Y	Y	N	Y	Y	N	Y	Y	N	8 (50.00)	Very low
Zhang *et al.* (2021)[11]	Y	PY	Y	PY	Y	Y	N	Y	Y	N	Y	Y	Y	Y	Y	Y	12 (75.00)	Very low
Jiang *et al.* (2023)[12]	Y	Y	Y	PY	Y	Y	N	Y	Y	N	Y	Y	Y	Y	Y	N	12 (75.00)	Very low
Zhong *et al.* (2023)[13]	Y	Y	Y	Y	Y	Y	N	Y	PY	Y	Y	Y	Y	Y	Y	Y	14 (87.50)	Very low

Note: [a]The key items of the AMSTAR-2.

Abbreviations: AMSTAR: A measurement tool to assess systematic reviews; N: No; PY: Partial yes; Y: Yes.

Table A3. Results of the PRISMA checklist

Section/topic	Items	Li *et al.* (2012)[1]	Zhang *et al.* (2014)[2]	Niu (2014)[3]	Zhang *et al.* (2014)[4]	Zhang and Liu (2017)[5]	Li *et al.* (2018)[6]	Huang *et al.* (2018)[7]	Wei *et al.* (2020)[8]	Wang *et al.* (2021)[9]	Zhang *et al.* (2021)[10]	Zhang *et al.* (2021)[11]	Jiang *et al.* (2023)[12]	Zhong *et al.* (2023)[13]
Title	1. Title	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Abstract	2. Structured summary	Y	PY	PY	Y	PY	Y	PY	PY	PY	Y	PY	PY	Y
Introduction	3. Rationale	PY	Y	Y	Y	PY	Y	Y	Y	Y	Y	Y	Y	Y
	4. Objectives	Y	Y	Y	Y	Y	Y	Y	Y	Y	PY	Y	Y	Y
Methods	5. Protocol and registration	N	N	N	N	N	Y	N	N	N	N	N	Y	Y
	6. Eligibility criteria	Y	Y	Y	PY	PY	Y	Y	Y	PY	PY	Y	Y	PY
	7. Information sources	N	PY	PY	PY	Y	PY	Y	PY	PY	PY	Y	PY	PY
	8. Search	N	Y	N	Y	N	N	N	N	N	Y	Y	Y	Y
	9. Study selection	Y	Y	Y	Y	Y	N	Y	PY	Y	N	Y	PY	Y
	10. Data collection process	N	Y	Y	Y	N	Y	N	PY	N	Y	Y	Y	Y
	11. Data items	PY	PY	PY	PY	PY	PY	PY	PY	PY	PY	PY	PY	PY
	12. Risk of bias in individual studies	Y	Y	Y	N	N	Y	Y	Y	Y	Y	Y	Y	Y
	13. Summary measures	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y
	14. Synthesis of results	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
	15. Risk of bias across studies	Y	Y	Y	Y	N	Y	N	N	Y	Y	Y	Y	Y
Results	16. Additional studies	N	Y	N	Y	N	N	Y	N	Y	N	Y	Y	Y
	17. Studies selection	Y	PY	Y	Y	Y	Y	Y	PY	PY	PY	Y	PY	Y
	18. Studies characteristics	PY	PY	Y	PY	PY	Y	Y	PY	PY	Y	Y	PY	PY
	19. Risk of bias within studies	Y	Y	Y	N	N	Y	N	Y	Y	Y	Y	Y	Y
	20. Results of individual studies	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

(Contd...)

Table A3. (*Continued*)

Section/topic	Items	Li *et al.* (2012)[1]	Zhang *et al.* (2014)[2]	Niu (2014)[3]	Zhang *et al.* (2014)[4]	Zhang and Liu (2017)[5]	Li *et al.* (2018)[6]	Huang *et al.* (2018)[7]	Wei *et al.* (2020)[8]	Wang *et al.* (2021)[9]	Zhang *et al.* (2021)[10]	Zhang *et al.* (2021)[11]	Jiang *et al.* (2023)[12]	Zhong *et al.* (2023)[13]
	21. Synthesis of results	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y
	22. Risk of bias across studies	Y	Y	Y	N	N	Y	N	Y	Y	Y	Y	Y	Y
Discussion	23. Additional analyses	N	Y	N	Y	N	N	Y	Y	Y	N	Y	Y	Y
Other information	24. Summary of evidence	N	N	Y	Y	N	Y	Y	N	Y	Y	Y	Y	Y
	25. Limitations	N	Y	N	N	Y	N	N	Y	N	Y	Y	Y	Y
	26. Conclusions	N	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y
	27. Funding	N	N	N	N	N	Y	N	N	N	N	Y	N	Y

Abbreviations: N: No; PRISMA: Preferred Reporting Items for Acupuncture Systematic Reviews; PY: Partial yes; Y: yes.

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ACCSCIENCE PUBLISHING

Advanced Neurology

ORIGINAL RESEARCH ARTICLE

Mechanistic investigation of *Xijiao Dihuang* decoction for ischemic stroke: A network pharmacology and *in vivo* validation study

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Abstract

Ischemic stroke is the most prevalent and severe form of cerebrovascular disease and a leading cause of significant neurological disability. As the primary cause of hospitalization for neurological disorders, it necessitates urgent and effective therapeutic interventions. *Xijiao Dihuang* decoction (XJDHT), a classical traditional Chinese medicinal formula, is traditionally known for its effects in heat clearing, detoxification, blood cooling, and blood stasis removing. Emerging evidence suggests its potential to promote neurological recovery following ischemic stroke; however, systematic evaluation remains limited. This study employed a systematic network pharmacology approach to elucidate the molecular mechanisms underlying XJDHT's therapeutic effects on ischemic stroke. LC-MS identified 24 bioactive compounds in XJDHT, using Traditional Chinese Medicine Systems Pharmacology and SwissTargetPrediction databases for target prediction. Disease targets for ischemic stroke were retrieved from GeneCards, Online Mendelian Inheritance in Man, Therapeutic Target Database, and Gene Expression Omnibus datasets. Intersection analysis found 424 overlapping targets between 690 compound targets and 3415 disease targets. Cytoscape and STRING platforms were used to construct networks and identify hub genes and critical modules. Functional enrichment analysis was conducted using the Database for Annotation, Visualization, and Integrated Discovery database. Network analysis identified core bioactive constituents, including paeoniflorin, albiflorin, benzoylpaeoniflorin, kaempferol, paeonol, ellagic acid, pinostrobin, β-sitosterol, and stigmasterol. Key therapeutic targets comprised phosphatidylinositol-4,5-bisphosphate 3-kinase regulatory subunit 1, SRC proto-oncogene, signal transducer and activator of transcription 3, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta, AKT serine/threonine kinase 1, epidermal growth factor receptor, estrogen receptor 1, Harvey rat sarcoma virus, and neuroblastoma RAS viral oncogene homolog. The Kyoto Encyclopedia of Genes and Genomes pathway enrichment revealed significant involvement in the mammalian target of rapamycin signaling pathway, mitogen-activated protein kinase cascade, phosphatidylinositol 3-Kinase-Protein Kinase B (PI3K-AKT) pathway, erythroblastic leukemia viral oncogene homolog signaling, and vascular endothelial growth factor-mediated angiogenesis. These findings suggest XJDHT exerts neuroprotective effects through coordinated modulation of inflammatory responses and cellular signaling cascades, exhibiting the characteristic therapeutic properties of a multicomponent, multitarget, and multipathway approach.

Keywords: Ischemic stroke; Traditional Chinese medicine; Network pharmacology; Micromolecular protein; Anti-inflammation; Antioxidation

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1. Introduction

Ischemic stroke, resulting from cerebral artery occlusion, constitutes one of the leading causes of chronic neurological disability worldwide. Despite the progress made in medical science, the development of effective therapeutic interventions to enhance functional recovery post-stroke remains an unmet clinical need with a significant socioeconomic burden.[1] Stroke management has entered a dynamic era of innovation, with current therapeutic paradigms revolutionized by two groundbreaking interventions: Intravenous thrombolysis and endovascular mechanical thrombectomy.[2] Additional strategies include antiplatelet aggregation therapy, lipid-lowering interventions, plaque stabilization, and comprehensive management of underlying conditions such as atrial fibrillation, hypertension, and diabetes mellitus.

According to traditional Chinese medicine (TCM) theory, the pathogenesis of stroke originates from the accumulation of endogenous pathogenic factors, including internal trauma, emotional overstimulation, improper diet, and chronic fatigue. These factors can lead to hepatic *Yang* hyperactivity, phlegm-heat retention, or *qi* deficiency with phlegm-dampness accumulation, ultimately causing disruption of wind regulation, imbalance of *qi*-blood circulation, and dysfunction of the meridian networks. This pathophysiological cascade contributes to cerebrovascular impairment through blood stasis or hemorrhagic extravasation.

Xijiao Dihuang decoction (XJDHT), a classical formula documented in the Tang Dynasty medical compendium *Medical Secrets of an Official* by Wang Tao, exhibits dual therapeutic effects: Heat clearing/detoxification and blood cooling/stasis dispelling. Studies have demonstrated that XJDHT promotes the proliferation, migration, and tube formation of endothelial cells by upregulating vascular endothelial growth factor (VEGF) and basic fibroblast growth factor, which together stimulate the release of matrix-degrading enzymes from endothelial cells, enhance cell migration ability, and promote neovascularization. Concurrently, XJDHT alleviates inflammatory responses associated with ischemic brain injury by inhibiting proinflammatory factors, thereby improving neurological deficits.[3] In addition to promoting vascular endothelial growth and exerting anti-inflammatory effects, XJDHT enhances the proliferation and differentiation of neural stem cells by upregulating glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor in middle cerebral artery occlusion (MCAO) rats, thereby supporting endogenous neural repair.[4] At the molecular level, XJDHT plays a neuroprotective role by inhibiting Toll-like receptor 4/myeloid differentiation factor 88/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway, which alleviates oxygen-glucose deprivation/reperfusion-induced inflammatory response and neuronal apoptosis.[5] Although research has demonstrated the neuroprotective, anti-inflammatory, and neural repair-promoting properties of XJDHT, its material basis underlying XJDHT's therapeutic effects and precise molecular remain inadequately characterized. To address this gap, the present study employed a network pharmacology approach to explore the mechanisms by which XJDHT facilitates neurological recovery.

Network pharmacology, grounded in systems biology principles, enables comprehensive analysis of biological networks and identification of key signaling nodes involved in multitarget drug actions. This discipline emphasizes multipathway regulation to enhance therapeutic efficacy when minimizing side effects, thereby improving clinical success rates and reducing drug development costs. The inherently multitarget nature of TCM formulations aligns well with the holistic and systematic framework network pharmacology, making this methodology particularly suitable for investigating the complex pharmacological mechanisms of Chinese herbal compounds.[6]

2. Materials and methods

2.1. Materials

Male Sprague–Dawley rats weighing 260 – 280 g were purchased from Hangzhou Medical College (SPF-grade, license No. SCXX [Zhe] 2019-0002) and housed in the animal facility of Nanjing Surui Co., Ltd. The facility maintained a temperature of 26 ± 2°C, relative humidity of 55 ± 5%, a 12 h/12 h light/dark cycle, and provided *ad libitum* access to food and water.

The XJDHT sample, consisting of 9 g of *Paeonia lactiflora*, 24 g of *Rehmannia*, 12 g of *Paeonia suffruticosa*, and 30 g of buffalo horn, was analyzed using mass spectrometry to obtain spectra and a composition table. The samples were sourced from the Pharmacy of Chinese Medicine at the First Clinical College of Nanjing University of Chinese Medicine. After decoction and filtration, the combined filtrate was concentrated using a rotary evaporator to achieve a final concentration of 1 g/mL. The resulting extract was stored at 4°C.

The reagents and instruments used in this study included:

(i) Edaravone injection (5 mL: Edaravone 10 mg and d-camphene 2.5 mg; Simcere Pharmaceutical Co., LTD, China);
(ii) 2,3,5-triphenyl-tetrazolium chloride (TTC) staining solution (2%; G3005; Solarbio, China);
(iii) Digital thermostatic water bath (HH-2; Shanghai

Yetuo, China);
(iv) Neuroblastoma RAS viral oncogene homolog (NRAS) polyclonal antibody (50 μL; Polymer Technology Group (PTG), America);
(v) 300 kDa global transcriptional coactivator (p300) antibody (50 μL; Affinity Biosciences, China);
(vi) LightCycler®480 real-time fluorescence quantitative polymerase chain reaction (qPCR) instrument (Roche Diagnostics, Germany);
(vii) RNA extraction reagent (Vazyme, China);
(viii) Reverse transcription (RT) Super Mix (Vazyme, China);
(ix) Fluorophore-conjugated secondary antibodies (Invitrogen, USA);
(x) Fluorescence microscope (Olympus IX71, Japan).

2.2. Model development

Twenty-four male Sprague–Dawley rats (280 – 330 g) were randomly divided into four groups (n = 6 per group): Control group, MCAO group, XJDHT group, and edaravone group. All rats were fasted for 12 h (with free access to water) prior to surgery. Anesthesia was induced via intraperitoneal injection of 10% chloral hydrate (3 mL/kg). The MCAO model was established using Longa's suture method. The anesthetized rats were secured in a supine position on a surgical board. After neck skin disinfection, a midline incision was made, and the muscles and fascia were bluntly dissected along the medial border of the sternocleidomastoid muscle to expose the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA). Sutures were pre-placed at the distal and proximal ends of the CCA and ECA. A microvascular clip was used to temporarily occlude the ICA. The CCA and ECA were ligated proximally, and a small incision was made 4 mm from the CCA bifurcation. A nylon monofilament (marked with correction fluid 19 mm from the tip) was inserted into the ICA and gently advanced using ophthalmic forceps until resistance was felt at the bifurcation of the ICA and ECA. The pre-placed sutures at the distal CCA and ICA were then tightened to secure the filament. After 90 min of ischemia, the filament was slowly withdrawn to initiate reperfusion. The muscle and skin layers were sutured sequentially, followed by iodophor disinfection. Post-operative intragastric administration and intraperitoneal injections were initiated 2 h after full recovery from anesthesia. Drug treatments were administered once daily at fixed intervals from post-operative days 2 – 7. On the 7th post-operative day, 2 h after administration, blood was collected under anesthesia, and the animals were sacrificed for brain collection. The brains of two mice in each group were fixed with 4% paraformaldehyde. The two rat brains were frozen at -80°C. The remaining brains were quickly harvested and transferred in a phosphate-buffered saline (PBS) solution at 0 - 4°C, then frozen in a refrigerator at -20°C for 30 min. The cerebellum, olfactory bulb, and lower brainstem were removed, and 2 mm-thick coronal brain slices were prepared. The slices were placed in appropriately sized containers, and TTC staining solution was added to completely immerse the tissue. The containers were incubated in water at 37°C in the dark for 20 – 30 min, with gentle shaking every 5 min to ensure thorough staining. After staining, the brain slices were rinsed with PBS solution for 3 – 5 min and then placed on a suitable background plate for photography and observation. Following photography, the slices were fixed in 4% paraformaldehyde. Brain slices from the control group appeared red, with no visible white infarcted area. Normal areas were stained red, while infarcted areas appeared white.

2.3. Determination of XJDHT composition

A compound library was established based on relevant literature and information on chemical compositions found in medicinal materials. Peakview SoftwareTM (AB Sciex) software was utilized for data analysis by inputting chemical formulas and molecular weight information. By combining secondary information fragments obtained from mass spectrometry with reference substances, compound databases, and related literature reports as references, the chemical compositions and structures of the compound preparations were further confirmed. Chromatograms derived from Peakview data were generated using Origin Pro 8.0 software to create a compound composition table for XJDHT.

2.4. XJDHT effective component selection

The Traditional Chinese Medicine Systems Pharmacology (TCMSP) database (https://tcmsp.91medicine.cn) and SwissADME database (http://www.swissadme.ch) were employed for pharmacochemical analysis, with a specific focus on oral bioavailability (OB), a critical pharmacokinetic parameter quantifying the rate and extent of systemic absorption. Potential bioactive compounds in the TCMSP database were systematically screened using stringent criteria: OB ≥ 30% and drug-likeness (DL) ≥0.18. Structural information for each compound was retrieved from the UniProt database (http://www.uniprot.org/uniprot/). Chemical identifiers from TCMSP were cross-referenced with SwissADME entries to obtain comprehensive molecular descriptors. Gastrointestinal (GI) absorption was evaluated as the percentage of compound absorption through intestinal epithelium, whereas blood–brain barrier (BBB) permeability was

categorized as positive or negative based on predictive models. SwissADME's five DL evaluation frameworks (Lipinski's, Ghose's, Veber's, Egan's, and Muegge's rules) were applied concurrently. For inclusion criteria, compounds were required to simultaneously satisfy: (i) High GI absorption classification, (ii) positive BBB permeability prediction, and (iii) compliance with all five established DL rules.

2.5. Active ingredients and disease targets screening

The chemical composition of the selected medicinal inputs was systematically obtained from two authoritative pharmacological databases: The SwissTargetPrediction target database (http://swisstargetprediction.ch) and the TCMSP database. These sources were cross-referenced to identify bioactive components meeting established target screening criteria. Subsequently, disease-associated genetic targets for ischemic stroke were comprehensively retrieved through multidatabase interrogation, including GeneCards (https://www.genecards.org/), Online Mendelian Inheritance in Man (OMIM, https://www.omim.org), and the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/), with strict inclusion parameters limited to experimentally validated human genes. Following duplicate removal and data consolidation, a curated set of ischemic stroke-related therapeutic targets was established. Intersectional analysis employing a Venn diagram methodology revealed statistically significant overlaps between pharmacologically active components and pathological targets, thereby identifying putative key molecular targets for TCM-based therapeutic intervention in ischemic stroke management.

2.6. Core target screening and analysis of the protein–protein interaction (PPI) network

PPI network construction and network topology analysis were performed using the STRING platform (https://cn.string-db.org/). Intersecting genes in *Homo sapiens* were identified with a confidence threshold set at 0.900 to ensure high-quality interactions, excluding unconnected gene nodes. The resulting protein interaction data were imported into Cytoscape 3.10.2 software, and network topology parameters were obtained using the "Network Analyzer" tool. The degree value represents the significance of each target, with higher values indicating greater importance of both drugs and targets. Core targets were ranked based on their degree values.

2.7. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis

GO and KEGG enrichment analyses were conducted using the Database for Annotation, Visualization, and Integrated Discovery (DAVID; https://david.ncifcrf.gov/). GO analysis employed a standardized ontological framework to systematically characterize target proteins across three hierarchical domains: Biological processes (BP), cellular components (CC), and molecular functions (MF). BP annotations encompassed drug treatment-related cellular activities, including proliferation regulation, signal transduction cascades, and metabolic pathways. CC annotations identified subcellular localization patterns, whereas MF annotations delineated molecular-level interactions and catalytic activities. Simultaneously, KEGG pathway analysis was performed to identify key signaling pathways associated with therapeutic mechanisms. The top 20 significantly enriched terms from each ontological category (BP, CC, and MF) and KEGG pathways were selected based on hypergeometric test p-values (<0.05). Visualization of the enrichment results, including multidimensional bubble charts and comparative histograms, was conducted using the Bioinformatics.com.cn platform (https://www.bioinformatics.com.cn/). This analytical approach enabled the comprehensive mapping of drug–target interactions to fundamental BP and disease-relevant signaling networks.

2.8. KEGG pathway filtering based on literature review

The present study systematically investigated molecular mechanisms in human biological pathways by querying 20 KEGG pathways through the KEGG database (https://www.kegg.jp/kegg/kegg1.html). Pathway selection criteria were established through a two-phase process: Initial prioritization of stroke-related terms via PubMed database searches (https://pubmed.ncbi.nlm.nih.gov/), followed by comprehensive analysis of existing literature to identify validated signaling pathways, protein interactions, and pathway crosstalk. Identified molecular components were subsequently re-examined in the KEGG database to map upstream–downstream regulatory relationships. Proteins demonstrating significant associations with active ingredient–disease interactions were prioritized for molecular docking simulations and experimental validation through rigorous selection protocols.

2.9. Messenger RNA transcript level quantification via quantitative real-time polymerase chain reaction

Real-time qPCR is a method that uses fluorescent chemicals to measure the total amount of product after each PCR cycle in a DNA amplification reaction. It allows for quantitative analysis of a specific DNA sequence in a test sample using internal or external reference standards. The messenger RNA (mRNA) expression level indirectly reflects potential protein synthesis. Proteins with significantly altered

mRNA transcription levels in the XJDHT group were selected for molecular docking and immunofluorescence staining. The specific steps were as follows: After cell lysis, 50 μL p300 antibody and 50 μL NRAS antibody were prepared. For sample processing and antibody premixing, a total RNA extraction reagent was used to extract mRNA from rat brain tissue. Complementary DNA (cDNA) was synthesized by reverse transcription using RT Super Mix to quantify the mRNA levels of each factor. PCR analysis of the diluted cDNA samples was performed using Fluorescent Quantitative PCR Mix and the LightCycler®480 II system.

2.10. Molecular docking

Molecular docking simulates the molecular recognition process between small-molecule ligands and macromolecular protein targets, with binding energy (affinity) serving as the primary quantitative metric for evaluating docking performance. Lower binding energy values indicate more favorable molecular interactions and more stable complex formation.

The experimental protocol comprised three principal phases: Ligand preparation, protein preparation, and docking implementation. Ligand structures of bioactive compounds were retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/) in Semantic Definition Format, followed by molecular optimization using PyMOL software for hydrogen atom addition and charge assignment employing the MMFF94 force field. Energy minimization procedures were subsequently performed to achieve stable molecular conformations, with final conversion to PDBQT format executed through AutoDockTools. Protein target selection from the RCSB Protein Data Bank (http://www.rcsb.org/) adhered to three critical criteria: (i) Biological relevance in disease pathology; (ii) presence of cocrystallized ligands; and (iii) preferential selection of crystal structures with minimized resolution values. Protein preparation involved ligand separation, hydrogen addition, and charge calculation via AutoDockTools, culminating in conversion to PDBQT format. Docking parameters were standardized using AutoDockTools to define grid box dimensions and coordinate centering. Molecular docking simulations were performed using AutoDock Vina, followed by affinity extraction and statistical analysis of binding energies for comparative evaluation.

2.11. Immunofluorescence staining

Immunofluorescence staining is a laboratory technique used to identify and localize specific biomolecules within cellular or tissue specimens through the utilization of fluorophore-conjugated antibodies, which exhibit high affinity for target epitopes, enabling precise spatial visualization through fluorescence microscopy. Proteins demonstrating significant expression alterations in qPCR analyses were subjected to immunofluorescence localization in rat cerebral tissue sections using Alexa Fluor 594-conjugated secondary antibodies. Confocal laser scanning microscopy facilitated detailed observation of protein distribution patterns within neuroanatomical structures. Quantitative fluorescence intensity measurements were acquired through standardized threshold analysis in ImageJ software (v1.53t; National Institutes of Health, US), with statistical data presented in comparative histogram format. Paraffin-embedded brain tissue sections were deparaffinized and rehydrated through graded alcohols, then fixed in 4% paraformaldehyde. Sections were permeabilized with 0.3% Triton X-100 for 5 min and blocked with bovine serum albumin for 30 min at room temperature to reduce non-specific binding. Subsequently, sections were incubated overnight at 4°C with primary antibodies against E1A binding protein P300 (EP300) and NRAS. After washing, sections were incubated with appropriate fluorophore-conjugated secondary antibodies (Invitrogen, USA) for 30 min at room temperature. Nuclear counterstaining was performed using 4',6-diamidino-2-phenylindole. Immunofluorescent signals for EP300 and NRAS were visualized using a fluorescence microscope (Olympus IX71, Japan) under appropriate filter settings.

3. Results

3.1. Observation of curative effect

As illustrated in Figure 1, rat brain tissue sections are categorized into four groups: (i) Control group, (ii) MCAO model group, (iii) XJDHT treatment group, and (iv) edaravone positive control group (abbreviated as edaravone). After treatment with XJDHT and edaravone, the area of white infarction was reduced. Table 1 presents the volume ratio of ischemic regions in brain tissue for each group. The volume ratio of the ischemic area was highest in the MCAO group, indicating successful model establishment. Blocking the middle cerebral artery blood flow in rats led to obvious ischemic cerebral infarction. The volume ratios in the XJDHT group were 22.40 and 10.64; notably, the value of 10.64 was much lower than that in the MCAO group, suggesting that XJDHT treatment has the potential to improve ischemia. In terms of efficacy, XJDHT appears to be superior to edaravone. Brain slices from the control group appeared red, with no visible white infarction areas. According to the TTC staining results, the proportion of infarction area to the total brain area was highest in the MCAO model group compared to the control group, while the proportions were significantly reduced in the XJDHT decoction and edaravone groups.

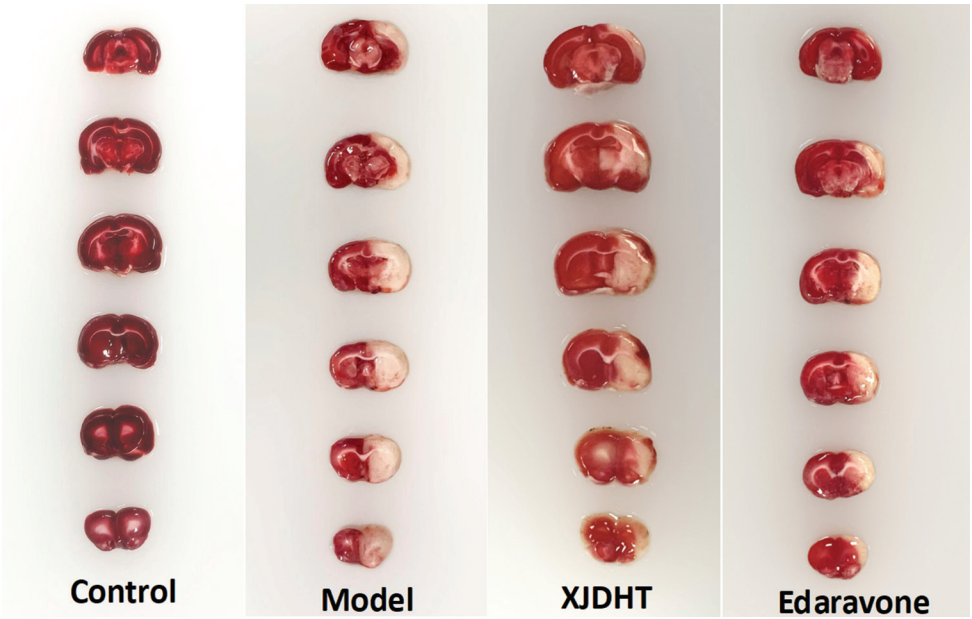


Figure 1. Schematic representation of the ischemic regions in rat brain tissue following treatment with each reagent
Abbreviation: XJDHT: *Xijiao Dihuang* decoction.

Table 1. Volume ratio of ischemic areas across groups

Group	Volume ratio of ischemic zone (%)	Mean value
1. Control	0	0
	0	
2. MCAO	33.94050568	36.80571236
	39.67091903	
3. Edaravone	13.31372863	17.03825104*
	20.76277344	
4. XJDHT	22.40604662	16.52421605*
	10.64238547	

Note: *$p<0.05$.
Abbreviations: MCAO: Middle cerebral artery occlusion; XJDHT: *Xijiao Dihuang* decoction.

3.2. Determination of drug components in XJDHT

Under optimized ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry conditions, representative ion chromatograms were obtained to define chromatographic separation parameters. The characteristic ion flux profiles in both positive and negative ionization modes are presented in Figure 2A and B. Through systematic analysis, XJDHT was found to contain 60 bioactive constituents, including arginine, gallic acid, quercetin, kaempferol, benzoyl paeoniflorin, stigmasterol, and sitosterol. These phytochemical components were systematically characterized based on mass spectral fragmentation patterns and database matching. The complete phytochemical profile, including retention times, molecular formulas, and mass-to-charge ratios, is comprehensively documented in Table 2.

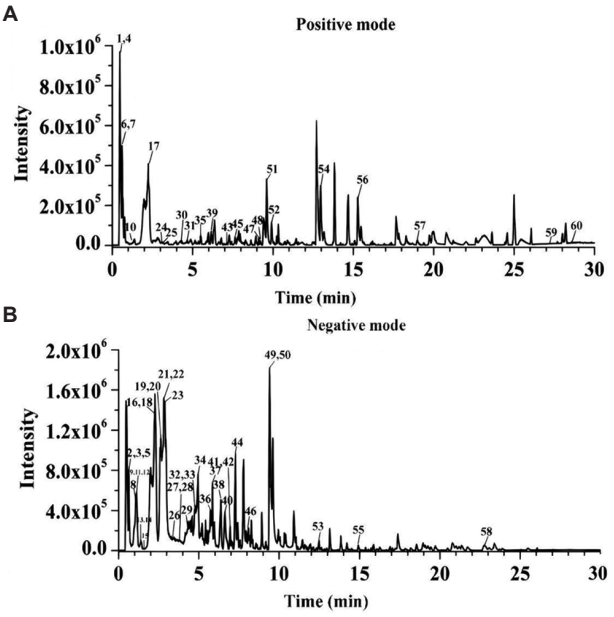


Figure 2. Basic peak ion current chromatogram of *Xijiao Dihuang* decoction in (A) positive ion mode and (B) negative ion mode

3.3. Effective component selection and sorting

In the TCMSP database, 24 components were selected, including albiflorin, paeoniflorin, berberine, and benzoyl paeoniflorin, based on the criteria of OB ≥ 30% and DL ≥ 0.18. In the SwissADME database, the conditions were

Table 2. Ingredient identification of X*ijiao Dihuang* decoction

t_R [min]	Formula	Theoretical MS	MS^1	MS/MS fragments	Identification
0.48	$C_6H_{14}N_4O_2$	175.1190	175.1188	116.0629	Arginine
0.60	$C_7H_6O_5$	169.0143	169.0144	125.0350,97.0320	Gallic acid
0.61	$C_{19}H_{26}O_{15}$	493.1199	493.1192	313.0686,169.0252,151.0049	Diglucosyl gallic acid
0.68	$C_9H_{11}NO_2$	166.0863	166.0860	120.0884,103.0616,91.0544	Phenprobamate
0.69	$C_{15}H_{24}O_9$	347.1348	347.1338	167.0716,149.0460,127.0359	Leonuride
0.71	$C_6H_6O_3$	127.0390	127.0389	109.0307,81.0352	3-hydroxy-2-methyl-4H-pyran-4-one
0.71	$C_9H_{17}NO_5$	220.1180	220.1176	184.0965,142.0858	D-pantothenic acid
0.84	$C_7H_6O_4$	153.0193	153.0191	109.04	Protocatechuic acid
1.01	$C_{23}H_{28}O_{13}S$	543.1178	543.1181	497.1585,259.0285,121.0296	Paeoniflorin sulfonate
1.16	$C_7H_6O_3$	139.0390	139.0390	121.03	Protocatechualdehyde
1.19	$C_{15}H_{14}O_6$	289.0718	289.0710	245.0819,179.0372,165.0200,151.0400,137.0249,109.0301	(+)-Catechin
1.29	$C_8H_8O_5$	183.0299	183.0296	168.0070,124.0224	Methyl gallate
1.47	$C_8H_8O_4$	167.0350	167.0345	123.05	Vanillic acid
1.5	$C_{23}H_{28}O_{11}$	525.1603	525.1629	479.2149,449.1445,327.1047,165.0558,121.0299	Albiflorin
1.67	$C_{29}H_{38}O_{16}$	687.2131	687.2131	519.1715,323.0983	Isomaltopaeoniflorin
2.20	$C_{20}H_{28}O_{12}$	459.1508	459.1500	165.06	Paeonolide
2.23	C_8H_8O	121.0648	121.0641	103.0534,91.0539	Acetophenone
2.24	$C_{16}H_{24}O_{10}$	375.1297	375.1272	345.1177,195.0671,139.0781	Debenzoyl paeoniflorin
2.61	$C_{23}H_{28}O_{11}$	525.1603	525.1609	449.1466,165.0561,121.0380	Paeoniflorin
2.67	$C_{15}H_{22}O_9$	345.1191	345.1188	183.1033,165.0917	Aucubin
2.84	$C_9H_{10}O_5$	197.0456	197.0451	169.0160,125.0256,124.0210	Ethyl gallate
2.98	$C_{15}H_{22}O_{10}$	361.1140	361.1153	199.0971,179.1052,161.0228	Monomelittin
3.02	$C_{20}H_{30}O_{12}$	461.1665	461.1627	135.04	Forsythoside E
3.19	$C_{10}H_{14}O_2$	167.1067	167.1063	149.0965,121.1033	6-pentyl-2H-pyran-2-one
3.21	$C_{10}H_{14}O$	151.1117	151.1112	133.0648,123.0798	Carvone
3.56	$C_{35}H_{46}O_{20}$	785.2510	785.2499	623.2235,161.0289	Purpureaside B
3.84	$C_{16}H_{24}O_8$	343.1398	343.1375	181.0498,151.0357	Mudanpioside F
3.86	$C_{24}H_{30}O_{15}S$	589.1233	589.1222	421.2731,258.9918	Mudanpioside E sulfite
4.17	$C_{14}H_6O_8$	300.9990	300.9987	229.02	Ellagic acid
4.42	$C_{10}H_{16}O$	153.1274	153.1269	135.1165,125.0228	Alpha-pinene oxide
4.61	$C_{27}H_{30}O_{15}$	595.1658	595.1658	287.05	Kaempferol 3-rutinoside
4.63	$C_{36}H_{48}O_{20}$	799.2666	799.2654	623.2224,175.0406	Jionoside A1
4.67	$C_{30}H_{32}O_{15}$	631.1668	631.1665	613.1577,491.1200	Galloyl paeoniflorin
5.19	$C_{23}H_{28}O_{12}$	495.1508	495.1500	165.0571,137.0616	Oxypaeoniflorin
5.41	$C_{21}H_{20}O_{12}$	465.1028	465.1009	303.1257,285.0742	Hyperoside
5.64	$C_{30}H_{32}O_{14}$	615.1719	615.1720	431.1383,281.0724,137.0323	Mudanpioside H
5.86	$C_{29}H_{36}O_{15}$	623.1981	623.1982	461.1672,161.0253	Forsythiaside
6.31	$C_{24}H_{30}O_{13}$	525.1614	525.1608	195.07	Mudanpioside E
6.32	C_9H_8O	133.0648	133.0647	115.0535,105.0694,	Trans-cinnamaldehyde
6.57	$C_{24}H_{30}O_{11}$	493.1715	493.1705	162.04	Paeonidanin
7.05	$C_{30}H_{38}O_{15}$	637.2138	637.2132	461.1699,175.0429	Jionoside D

(Cont'd...)

Table 2. *(Continued)*

tR [min]	Formula	Theoretical MS	MS^1	MS/MS fragments	Identification
7.07	$C_{37}H_{36}O_{19}$	783.1778	783.1769	465.14	3',6'-di-O-gal-loylpaeoniflorin
7.17	$C_{20}H_{17}NO_4$	336.1230	336.1231	320.0913,306.0717,292.0950,278.0809	Berberine
7.31	$C_{18}H_{24}O_{14}$	463.1093	463.1068	403.0879,373.0980,343.0660	Mudanoside B
7.62	$C_{15}H_{10}O_7$	303.0499	303.0496	257.04	Quercetin
8.04	$C_{17}H_{18}O_6$	317.1031	317.1019	121.03	Paeoniflorgenone
9.02	$C_9H_{10}O_3$	167.0703	167.0700	149.0589	Paeonol
9.15	$C_{15}H_{10}O_6$	287.0550	287.0549	165.02	Kaempferol
9.42	$C_{23}H_{26}O_{10}$	507.1497	507.1488	461.1482,431.1359,177.0558	Lactiflorin
9.43	$C_{23}H_{28}O_{11}$	525.1603	525.1602	479.1533,283.0821	Mudanpioside I
9.60	$C_{30}H_{32}O_{12}$	585.1967	585.1973	319.1251,267.0928	Benzoyl paeoniflorin
9.90	$C_9H_{10}O$	135.0804	135.0804	135.0793,107.0497	2,4-dimethylbenzaldehyde
12.47	$C_{15}H_{12}O_4$	255.0663	255.0648	227.0775,213.0549	Pinocembrin
12.96	$C_{18}H_{39}NO_3$	318.3003	318.3002	300.2897,256.2716,212.2369	Phytosphingosine
14.90	$C_{15}H_{10}O_5$	269.0456	269.0449	241.05	Apigenin
15.38	$C_{18}H_{30}O_2$	279.2319	279.2316	149.0226	β-elaeostearic acid
18.92	$C_{30}H_{46}O_4$	471.3469	471.3470	453.3325,435.3248,423.3230,407.3308,389.3201	18-β-glycyrrhetinic acid
22.8	$C_{30}H_{48}O_3$	455.3531	455.3523	-	Mairin
27.2	$C_{29}H_{50}O$	415.3934	415.3923	-	Sitosterol
28.8	$C_{29}H_{48}O$	395.3672	395.3666	257.2254,161.1316	Stigmasterol

Abbreviations: MS: Mass spectrometry; t_R: Retention time.

set to "high" GI absorption, "yes" for BBB permeability, and at least three "yes" in the five DL evaluation frameworks (Lipinski, Ghose, Veber, Egan, and Muegge), resulting in 12 components, including paeonol, carvone, phenprobamate, and pinocembrin. After deduplication of results from both databases, 24 potential active components of XJDHT were obtained: (+)-Catechin, albiflorin, paeoniflorin, ellagic acid, 2,4-dimethylbenzaldehyde, paeonol, trans-cinnamaldehyde, alpha-pinene oxide, carvone, phenprobamate, acetophenone, protocatechualdehyde, 6-pentyl-2h-pyran-2-one, 3-hydroxy-2-methyl-4h-pyran-4-one, stigmasterol, sitosterol, mairin, pinocembrin, benzoyl paeoniflorin, lactiflorin, kaempferol, paeoniflorgenone, quercetin, and berberine.

3.4. Screening of active ingredients and disease targets

The selected chemical components were input into the SwissTargetPrediction and TCMSP databases to screen for compound targets, resulting in 690 active component targets after the removal of duplicates. A search using the term "ischemic stroke" was conducted in GeneCards, OMIM, therapeutic target (Databasehttps://db.idrblab.net/ttd/), and GEO databases, restricted to the species *Homo sapiens* and including only experimentally validated genes, resulting in 3573 ischemic stroke-related disease targets. A Venn diagram was constructed to identify overlapping targets, revealing 424 intersection targets between active components and ischemic stroke, as shown in Figure 3.

3.5. Protein interaction analysis and "component–target" network construction

The STRING platform was used to obtain the protein interaction relationships. The resulting Tab-separated Values file was imported into Cytoscape3.10.2 software to generate the PPI map. Based on the degree value, the top 20 core targets were identified: Phosphatidylinositol-4,5-bisphosphate 3-kinase regulatory subunit 1 (PIK3R1), SRC proto-oncogene (SRC), signal transducer and activator of transcription (STAT) 3, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta (PIK3CB), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta (PIK3CD), AKT serine/threonine kinase 1 (AKT1), epidermal growth factor receptor (EGFR), mitogen-activated protein kinase (MAPK)1, heat shock protein 90 alpha class a member 1 (HSP90AA1), protein tyrosine phosphatase non-receptor

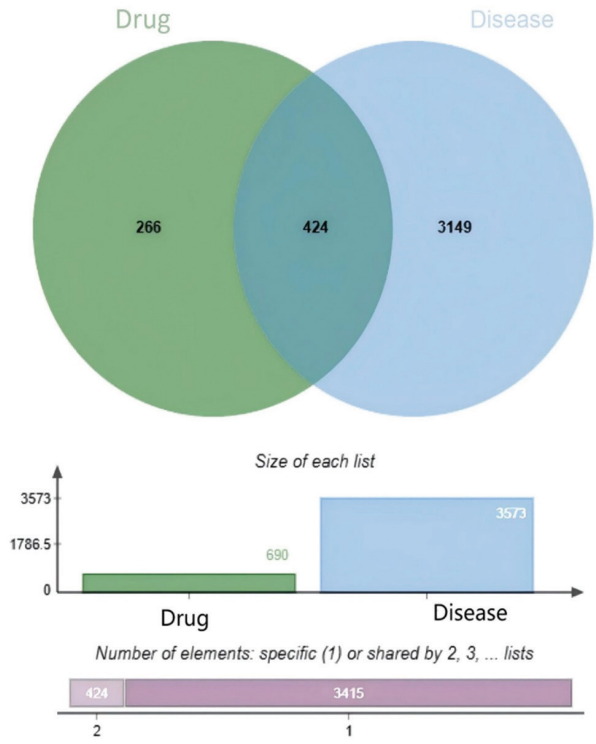


Figure 3. Intersection of effective components and ischemic stroke-related targets
Note: Green represents active drug ingredients, and blue represents the disease (ischemic stroke).

type 11, MAPK3, estrogen receptor 1 (ESR1), NRAS, Harvey rat sarcoma virus (HRAS), EP300, phospholipase C gamma 1, protein kinase A catalytic subunit alpha, MAPK8, and protein tyrosine kinase 2, as shown in Figure 4. Using the 24 active components and their associated targets, a "component–target" network diagram was constructed using Cytoscape3.10.2 software, as shown in Figure 5.

3.6. GO and KEGG enrichment analysis

The DAVID database was used to perform GO and KEGG functional enrichment analysis of the BP related to the intersection targets of active components and disease, in order to better understand the pharmacological blood-activating effect of XJDHT in the treatment of ischemic stroke. The following results were obtained, as shown in Figure 6A-C.

A total of 184 BP were associated with the 424 targets. These include phosphorylation, EGFR signaling pathway, signal transduction, insulin receptor signaling pathway, protein kinase B signaling, insulin-like growth factor receptor signaling pathway, cellular response to cadmium ion, protein phosphorylation, cellular response to reactive oxygen species (ROS), phosphatidylinositol-mediated signaling, T cell receptor signaling pathway, and T cell costimulation, among others.

Twenty-two CCs are involved in target interactions, including cytosol, plasma membrane, phosphatidylinositol 3-kinase (PI3K) complex class IA, PI3K complex, perinuclear region of differentiation, glutamatergic synapse, nucleoplasm, nucleus, and focal adhesion.

Forty-four MF were identified, including nitric oxide synthase regulator activity, protein phosphatase binding, adenosine triphosphate binding, protein kinase binding, kinase activity, phosphotyrosine binding, phosphatidylinositol-4,5-bisphosphate 3-kinase activity, 1-phosphatidylinositol-4-phosphate 3-kinase activity, protein kinase activity, and protein serine/threonine kinase activity.

KEGG pathways enrichment analysis showed that the 424 targets were involved in 147 major metabolism and signal transduction pathways, including endocrine resistance, proteoglycans in cancer, growth hormone synthesis, secretion and action, erythroblastic leukemia viral oncogene homolog (ErbB) signaling pathway, prolactin signaling pathway, EGFR tyrosine kinase inhibitor resistance, thyroid hormone signaling pathway, programmed death-ligand 1 expression and programmed cell death protein 1 checkpoint pathway in cancer, VEGF signaling pathway, and estrogen signaling pathway. These results are shown in Figure 7. The active ingredients, core targets, and core pathways are presented together in Figure 8, and the pathways with their corresponding numbers are listed in Table 3.

3.7. Selection of proteins for quantitative real-time polymerase chain reaction and expression analysis in rat brain tissue with ischemic stroke

Among the top 20 KEGG-related pathways, those with high research frequency and relatively comprehensive experimental validation included the VEGF signaling pathway, estrogen signaling pathway, lipid and atherosclerosis, and chemical carcinogenesis—receptor activation. Based on detailed KEGG pathway diagrams, signaling pathways potentially involved in ischemic stroke include the PI3K–protein kinase B (AKT) signaling pathway, MAPK signaling pathway, ErbB signaling pathway, mammalian target of rapamycin (mTOR) signaling pathway, and VEGF signaling pathway. Associated proteins include MAPK3, PIK3CA, PIK3CD, AKT1, MAPK1, HRAS, PIK3R1, PIK3CB, NRAS, SRC, EGFR, ESR1, HSP90AA1, STAT3, and EP300. After ischemic stroke model rats were treated with different reagents, the expression of related proteins in the brain tissue was detected by fluorescence qPCR to assess the

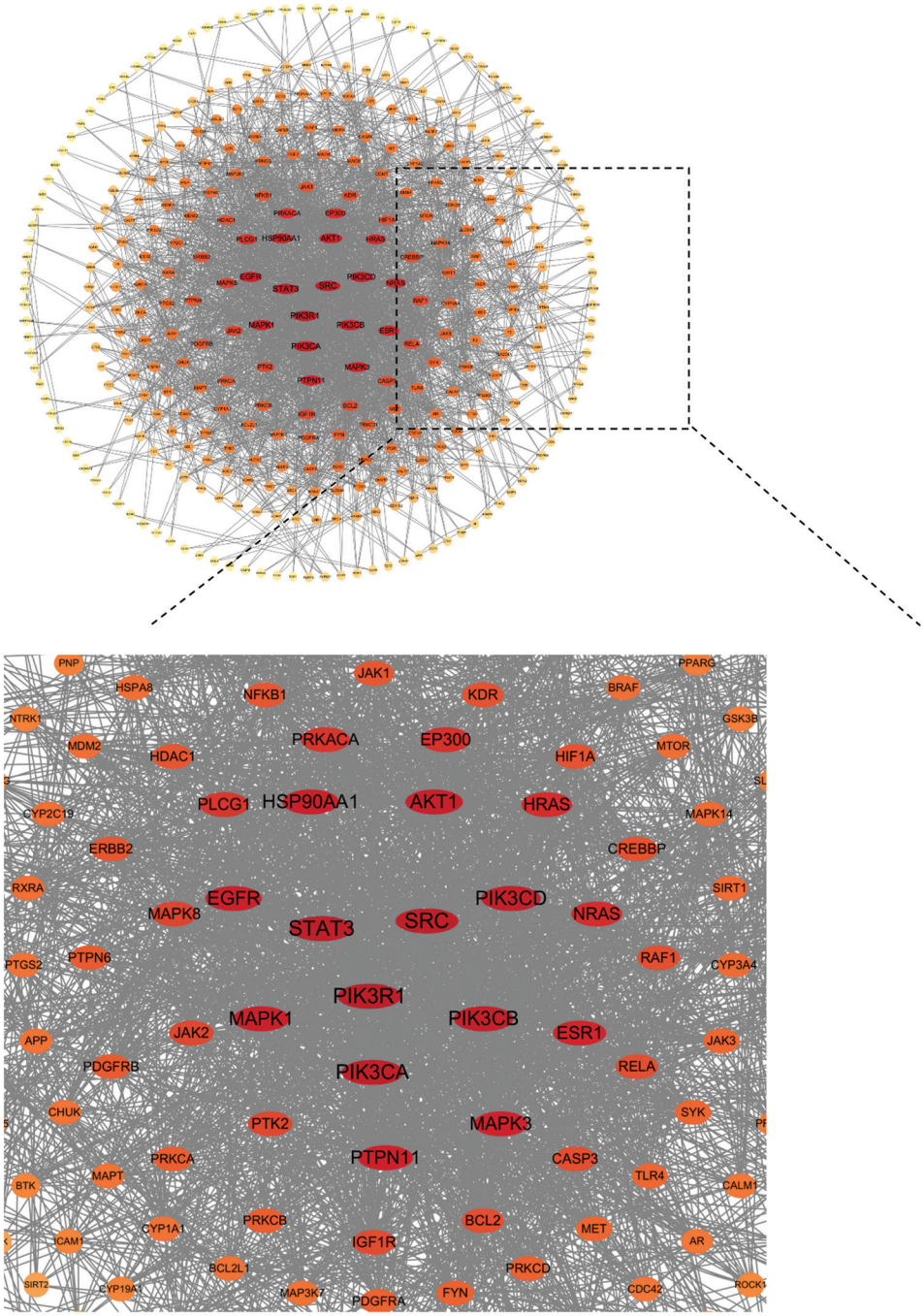


Figure 4. Protein–protein interaction network diagram
Note: Larger icons and deeper colors indicate protein with higher degree values.

therapeutic effect of XJDHT. Proteins such as MAPK3, PIK3CA, PIK3CD, AKT1, MAPK1, PIK3R1, PIK3CB, SRC, HSP90AA1, and STAT3 have been extensively studied. To explore new therapeutic mechanisms, five proteins (EP300, NRAS, EGFR, HRAS, and ESR) were selected for further investigation of their expression trends. Changes in protein levels were compared to the model group. For the HRAS protein, MCAO increased HRAS levels, while XJDHT and edaravone treatment decreased them; only the difference between the XJDHT group and the model group was statistically significant ($p<0.05$). For EGFR, MCAO and XJDHT decreased EGFR levels, whereas edaravone treatment increased EGFR levels; only the difference between the model group and

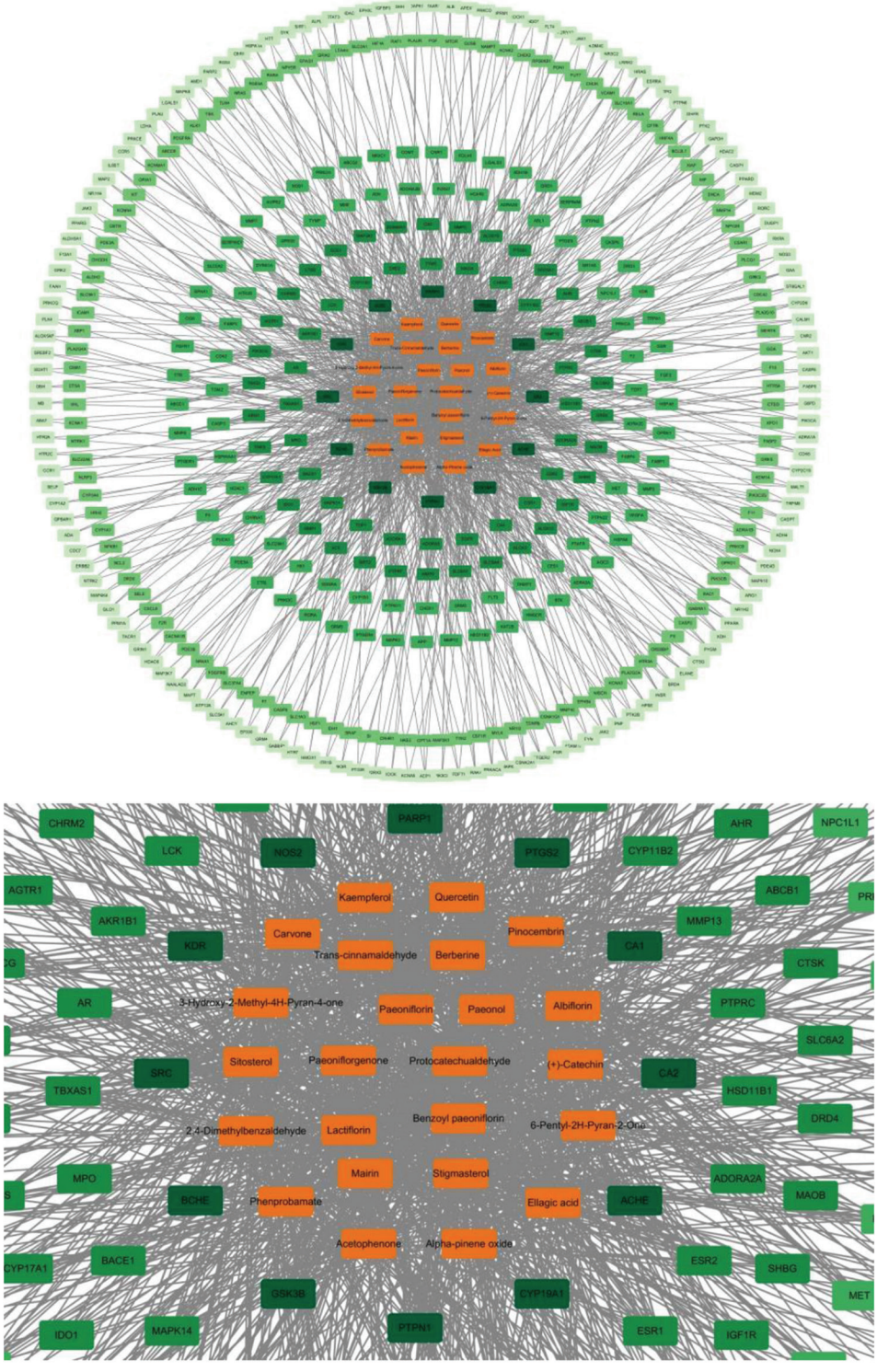


Figure 5. Component–target network diagram
Notes: Orange nodes in the center represent components, while green nodes on the periphery represent targets; Darker green indicates a higher degree value for the corresponding target.

the XJDHT group was statistically significant ($p<0.05$). For EP300, MCAO increased EP300 levels, while both treatments decreased them; the difference was statistically significant only in the XJDHT treatment group ($p<0.05$).

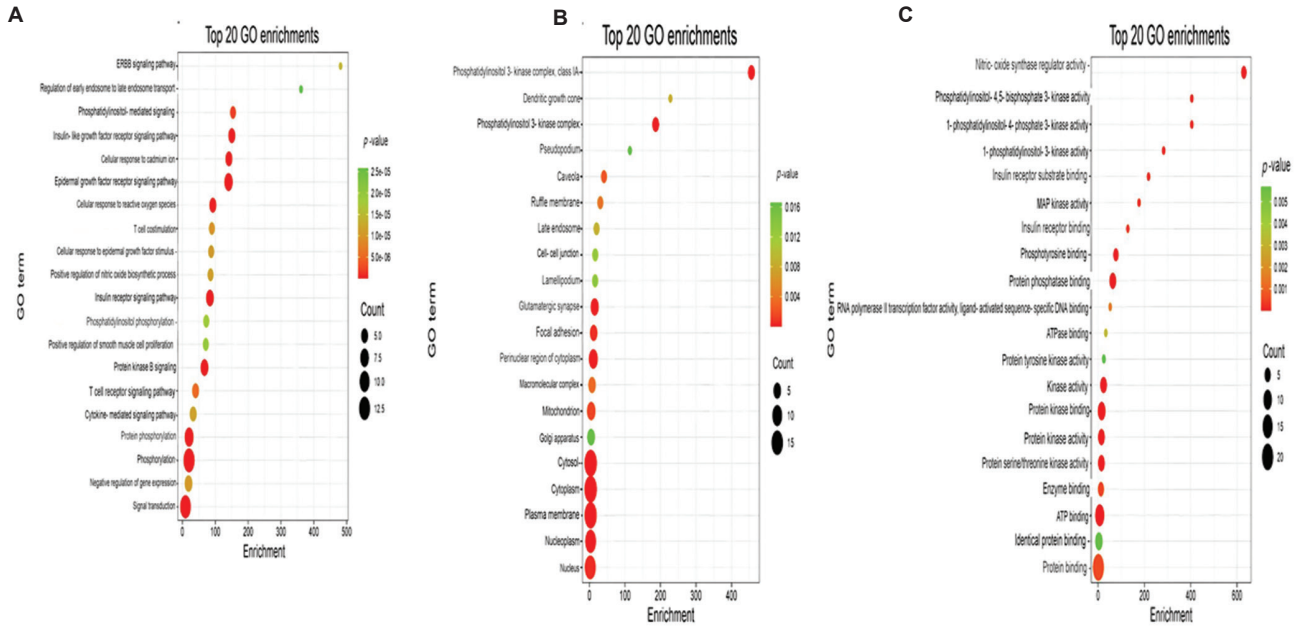


Figure 6. The top 20 Gene Ontology (GO) enrichments of (A) biological processes, (B) cellular components, and (C) molecular functions
Abbreviations: ATP: Adenosine triphosphate; ERBB: Erythroblastic Leukemia Viral Oncogene Homolog; MAP: Mitogen-activated protein kinase.

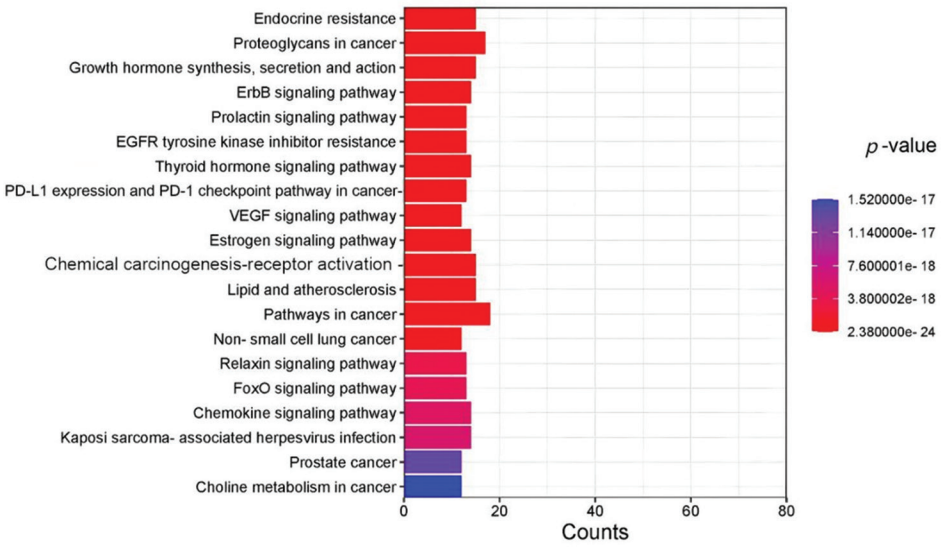


Figure 7. Top 20 Kyoto Encyclopedia of Genes and Genomes pathways
Notes: Each bar represents the number of genes associated with each pathway; A stronger correlation is indicated by a redder color.
Abbreviations: EGFR: Epidermal growth factor receptor; FoxO: Forkhead box O; PD-L1: Programmed death-ligand 1; PD-1: Programmed cell death protein 1; VEGF: Vascular endothelial growth factor.

For NRAS, MCAO increased NRAS levels, and XJDHT and edaravone decreased them, with significance only in the XJDHT treatment group ($p<0.05$). For ESR1, MCAO increased ESR1 levels, while both treatments decreased them; again, only the change in the XJDHT group was statistically significant ($p<0.05$).

Edaravone plays a neuroprotective role by scavenging free radicals and reducing oxidative stress damage to brain tissue. Studies have shown that edaravone can protect the integrity of cerebral microvessels and inhibit the overexpression of matrix metalloproteinase-9, thereby reducing vascular endothelial injury after cerebral ischemia.

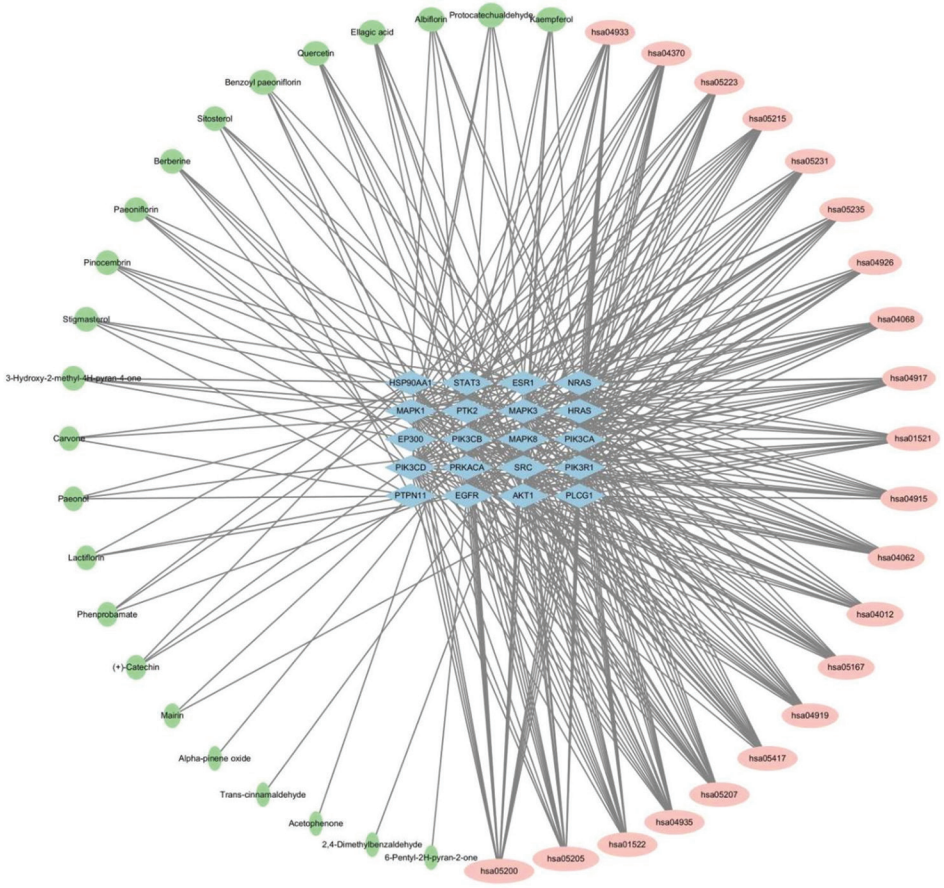


Figure 8. Relationship diagram of core components, targets, and pathways
Note: Blue represents core targets, green represents core components, and pink represents core Kyoto Encyclopedia of Genes and Genomes pathways.

In addition, edaravone delays neuronal death and improves neurological function recovery after cerebral ischemia. The related expression trends are shown in Figure 9.

3.8. Molecular docking

The qPCR results showed that changes in NRAS and EP300 proteins in the XJDHT group were more pronounced than those in the other groups. In addition, existing research is related to EP300 and NRAS; hence, the chemical components acting on these two proteins were selected for molecular docking. Molecular docking results showed that the core active compound albiflorin had a good binding affinity with NRAS, with a minimum molecular binding capacity of −8.8 kcal/mol. Hydroxy-2-methyl-4H-pyran-4-one and protocatechuic aldehyde (PCA) exhibited good binding affinity with EP300, with a minimum molecular binding capacity of −5.5 kcal/mol and −6.2 kcal/mol, respectively. Details of the optimal docking modes of hydroxy-2-methyl-4H-pyran-4-one and PCA from XJDHT with EP300 are shown in Figure 10.

3.9. Immunofluorescence staining

The immunofluorescence staining results of target proteins NRAS and EP300 in rat brain tissue showed that the average intensity of red fluorescence in the XJDHT group was lower than that in the control, model, and edaravone groups. These results indicate that the active components of XJDHT reduced the levels of NRAS and EP300 in brain tissue. The immunofluorescence staining results of the two target proteins are shown in Figure 11A and B. The EP300 level in the model group was significantly higher than in the control group, indicating that the model treatment had a significant impact on EP300 expression. The EP300 levels in the edaravone and the XJDHT groups were lower than those in the model group, indicating that the edaravone and XJDHT treatments had an inhibitory effect on the elevated EP300 expression in the model group. Similarly, the NRAS level in the model group was higher than that in the control group, with a significant difference, indicating that the model treatment increased NRAS expression. The NRAS levels in the edaravone and XJDHT groups

Table 3. Core KEGG pathways and their corresponding abbreviations

Abbreviation	Core KEGG pathway
hsa05200	Pathways in cancer
hsa05417	Lipid and atherosclerosis
hsa01521	EGFR tyrosine kinase inhibitor resistance
hsa04933	AGE-RAGE signaling pathway in diabetic complications
hsa04066	HIF-1 signaling pathway
hsa05205	Proteoglycans in cancer
hsa04080	Neuroactive ligand-receptor interaction
hsa05215	Prostate cancer
hsa04020	Calcium signaling pathway
hsa04014	Ras signaling pathway
hsa05167	Kaposi sarcoma-associated herpesvirus infection
hsa05161	Hepatitis B
hsa04024	cAMP signaling pathway
hsa05230	Central carbon metabolism in cancer
hsa04370	VEGF signaling pathway
hsa04151	PI3K–AKT signaling pathway
hsa05207	Chemical carcinogenesis–receptor activation
hsa05235	PD-L1 expression and PD-1 checkpoint pathway in cancer
hsa05212	Pancreatic cancer
hsa01522	Endocrine resistance

Abbreviations: AGE: Advanced glycation end-products; AKT: Protein kinase B; cAMP: Cyclic adenosine monophosphate; EGFR: Epidermal growth factor receptor; HIF-1: Hypoxia-inducible factor 1; PD-L1: Programmed death-ligand 1; PD-1: Programmed cell death protein 1; PI3K: Phosphatidylinositol 3-kinase; RAGE: Receptor for advanced glycation end-products; VEGF: Vascular endothelial growth factor; KEGG: Kyoto Encyclopedia of Genes and Genomes.

were lower than those in the model group, suggesting that edaravone and XJDHT treatments might inhibit the elevated NRAS expression in the model group.

4. Discussion

China has experienced an 86.0% increase in ischemic stroke incidence since 1990, currently accounting for 3.94 million new cases annually.[7] Researchers have demonstrated that XJDHT can significantly improve neurological function in patients[8] or mice suffering from ischemic stroke in both clinical trials and basic experiments.[9] The therapeutic efficacy rate of XJDHT in stroke patients has been shown to reach 98.0%, according to comprehensive clinical evaluations.[8] Through the integration of network pharmacology approaches with systematic experimental validation, this study elucidates the therapeutic efficacy and underlying molecular mechanisms of XJDHT in the treatment of ischemic stroke.

This study identified 24 active ingredients and 690 therapeutic targets associated with XJDHT, along with 3573 ischemic stroke-related targets retrieved from public databases. Through cross-analysis, 424 overlapping targets were identified between XJDHT components and ischemic stroke pathology. Network pharmacological analysis revealed 10 core bioactive compounds: Ellagic acid, kaempferol, pinocembrin, sitosterol, stigmasterol, PCA, carvone, albiflorin, paeoniflorin, and benzoyl paeoniflorin.

The qPCR validation results prompted further investigation into EP300 and NRAS targets, along with their corresponding active constituents: Hydroxy-2-methyl-4H-pyran-4-one, PCA, and albiflorin, for subsequent mechanistic exploration.

EP300, also known as histone acetyltransferase p300, is a nuclear enzyme encoded by the *EP300* gene.[10] EP300 overexpression is associated with prostate cancer, breast cancer, hepatocellular carcinoma, and non-small cell lung cancer and is therefore considered a potential therapeutic target in oncology.[11] It regulates gene expression through the acetylation of histones and other transcription factors, thereby affecting chromatin structure and function and contributing to fibrosis development, making it a potential target for anti-fibrosis therapies.[12] In neurological diseases, EP300 is recruited to specific gene enhancer regions by interacting with the transcription factor TFAP2β in the core transcriptional regulatory circuit of neuroblastoma, thereby regulating the expression of genes associated with the malignant phenotype of neuroblastoma cells.[13] A carboxypeptidase vitelloin-like protein promotes glioma progression by inhibiting the signal STAT 1 pathway through interaction with the Bruton's tyrosine kinase/p300 axis.[14]

Accumulating evidence from recent investigations indicates that EP300 serves as a pivotal regulator in cellular survival mechanisms, particularly through transcriptional modulation of apoptosis-related genes and chromatin remodeling processes.[13] Its association with stroke pathogenesis is manifested through regulatory effects on inflammatory responses and autophagy mechanisms. Under ischemic conditions, mTORC1 inhibition induces unc-51-like autophagy activating kinase 1 phosphorylation, thereby enhancing autophagic flux. This activated autophagy pathway facilitates the degradation of impaired cellular constituents during cerebral ischemia, demonstrating a critical neuroprotective mechanism.[15] EP300 can acetylate the raptor protein in the mTORC1 complex; acetylation of the raptor facilitates the binding of mTORC1 to lysosomes, thereby promoting its activity and inhibiting autophagy.[16] Furthermore, EP300 exerts inhibitory effects on autophagy initiation by mediating the acetylation of the PI3K catalytic

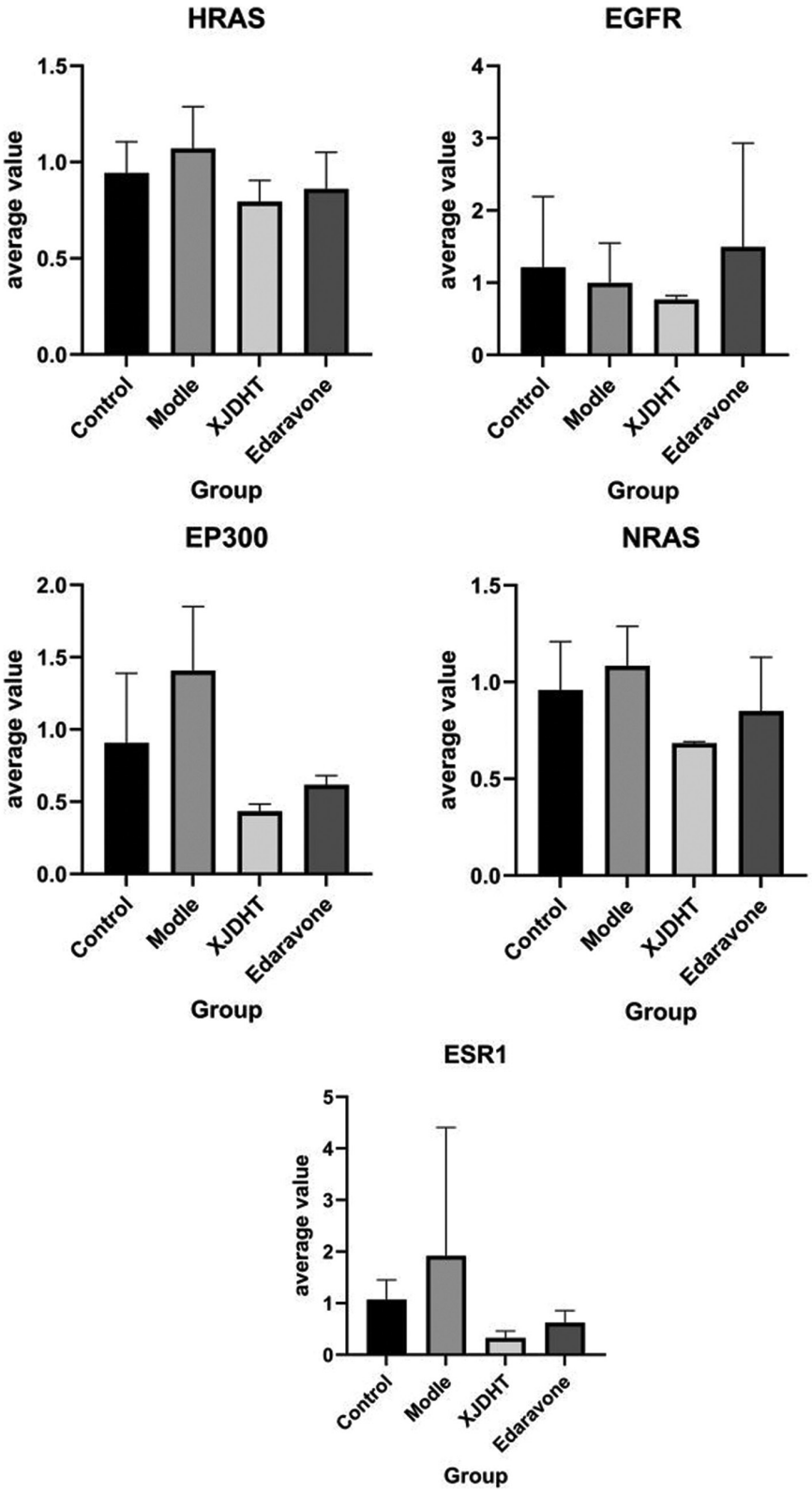


Figure 9. Expression trends of *binding protein P300*, *NRAS*, *EGFR*, *HRAS*, and *ESR1* detected with quantitative polymerase chain reaction
Abbreviations: EGFR: Epidermal growth factor receptor; ESR1: Estrogen receptor 1; HRAS: Harvey rat sarcoma virus; NRAS: Neuroblastoma RAS viral oncogene homolog.

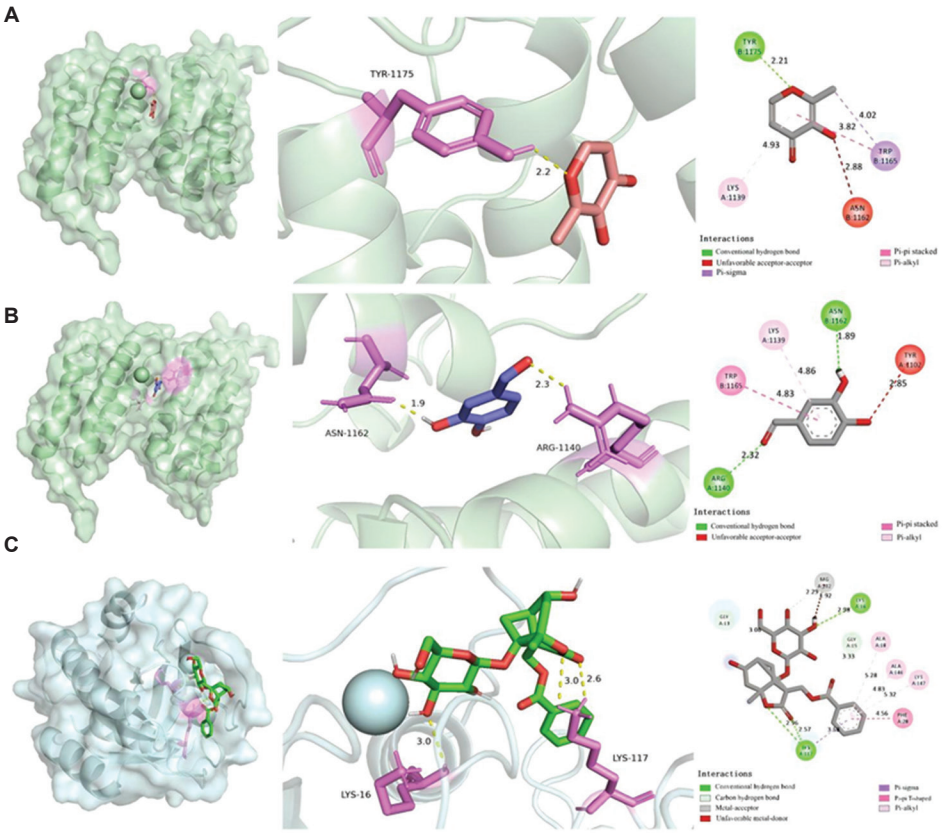


Figure 10. Molecular docking of hydroxy-2-methyl-4H-pyran-4-one and protocatechuic aldehyde from *Xijiao Dihuang* decoction with binding protein P300 (EP300). (A) Schematic representation of EP300 binding to hydroxy-2-methyl-4H-pyran-4-one. (B) Schematic representation of EP300 binding to protocatechuic aldehyde. (C) Schematic representation of the neuroblastoma RAS viral oncogene homolog binding to albiflorin.
Abbreviations: ALA: Alanine; ARG: Arginine; ASN: Asparagine; GLY: Glycine; MG: Magnesium ion; PHE: Phenylalanine; LYS: Lysine; TYR: Tyrosine; TRP: Tryptophan.

subunit type 3 (PIK3C3/VPS34) complex.[17] PIK3C3 affects the process of neuroinflammation by regulating autophagy in microglia.[18] During ischemic injury in neuronal cells, the autophagic pathway is initiated through activation of microtubule-associated protein 1 light chain 3. This critical autophagy marker undergoes proteolytic cleavage and lipidation to form the LC3-II isoform, which subsequently anchors to autophagosomal membranes through covalent conjugation. This molecular transformation facilitates autophagosome biogenesis and maturation during cellular stress responses.[19] EP300 inhibits autophagosome biogenesis by suppressing the LC3 lipidation process.[20] Following ischemic stroke, syntaxin 17 (STX17) facilitates cellular clearance of damaged organelles and misfolded proteins by mediating autophagosome–lysosome fusion, thereby mitigating ischemia-induced neuronal damage via enhanced autophagic flux.[21] EP300 impairs autophagosome–lysosome fusion through acetylation-mediated modulation of STX17.[22] Experimental evidence demonstrates that both aspirin and its bioactive metabolite salicylate elicit autophagic responses through selective inhibition of EP300 acetyltransferase activity.[23]

In addition, EP300 promotes the development of inflammation through transcriptional regulation by controlling the expression of cytokines and chemokines and activating the function of inflammatory cells. The increased expression of p53 protein in ischemic regions promotes neuronal apoptosis.[24] Acetylation of p53 by EP300 enhances its transcriptional activity and protects it from degradation.[25] During the pathogenesis of ischemic stroke, tumor necrosis factor (TNF)-α initiates a cascade of inflammatory responses by stimulating the synthesis and release of secondary inflammatory mediators. This cytokine-mediated process facilitates the recruitment and subsequent activation of immunocompetent cells within the neurovascular unit, thereby exacerbating secondary neuronal injury through amplified inflammatory mechanisms.[26] In addition, TNF-α can lead to the destruction of the BBB and increase the permeability of brain tissue, allowing more inflammatory cells and substances to enter the brain and aggravate the

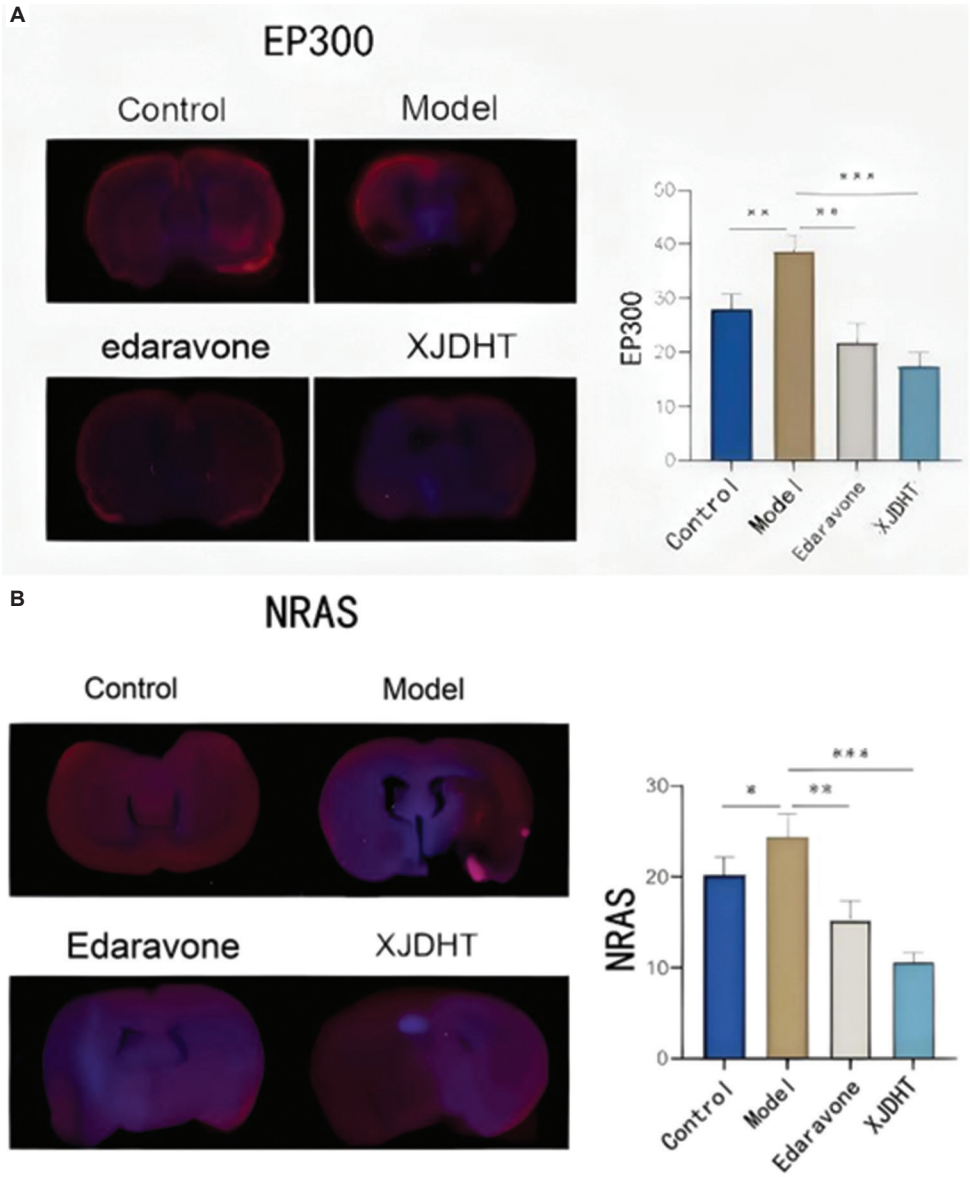


Figure 11. Immunofluorescence staining of NRAS and binding protein P300 (EP300) in brain tissue. (A) Distribution and intensity of EP300 staining in brain tissue sections from each group. (B) Distribution and intensity of NRAS staining in brain tissue sections from each group.
Notes: *p<0.05; **p<0.01; ***p<0.001
Abbreviations: NRAS: Neuroblastoma RAS viral oncogene homolog; XJDHT: *Xijiao Dihuang* decoction.

injury.[27] EP300 potentiates the transcriptional activity of TNF-α both through direct interactions with promoter-bound transcription factors and via dual mechanisms involving chromatin remodeling[12] or regulation of the NF-κB signaling pathway.[28]

Hydroxy-2-methyl-4H-pyran-4-one (commonly termed maltol), an EP300-modulating compound, demonstrates significant neuroprotective effects in stroke intervention. Experimental evidence reveals that maltol administration exerts dual antioxidant mechanisms: Not only does it attenuate oxidative stress through marked reductions in ROS generation and malondialdehyde accumulation in cerebral tissues, but it also enhances antioxidant enzyme activity. Mechanistic studies further indicate that maltol-mediated neuroprotection involves potentiation of the PI3K/AKT/nuclear factor erythroid 2-related factor 2 (NRF2) signaling axis, as evidenced by increased phosphorylation levels of PI3K, AKT, and NRF2.[29] Regarding its anti-inflammatory properties, maltol has been shown to significantly inhibit the production of pro-inflammatory mediators through downregulation

of the PI3K/AKT/NF-κB signaling pathway.[30] Maltol suppresses the phosphorylation of p38 proteins, effectively blocking activation of the p38 MAPK signaling pathway and consequently mitigating cellular inflammatory responses.[31]

Comprehensive analysis of the forkhead box O signaling pathway mapping via the KEGG database revealed that both PI3K–AKT and MAPK signaling pathways occupy upstream regulatory positions relative to EP300. The PI3K/AKT signaling axis modulates EP300 activity through targeted phosphorylation mechanisms. Specifically, AKT-mediated phosphorylation modifies the binding dynamics between EP300 and associated transcription factors, consequently inducing transcriptional regulation of downstream genes.[32] Following activation of the Janus kinase–STAT signaling cascade, STAT transcription factors undergo nuclear translocation and form functional complexes with the EP300 transcriptional coactivator, thereby orchestrating the transcriptional activation of cognate target genes.[33] The MAPK signaling pathway catalyzes the phosphorylation of EP300 through kinase activation. This post-translational modification potentiates the transcriptional coactivator function of EP300, thereby inducing transcriptional activation of downstream target genes.[34]

These findings suggest that maltol may exert therapeutic effects against ischemic stroke through a mechanism involving the inhibition of EP300 phosphorylation via dual modulation of the PI3K/AKT and MAPK signaling pathways. This regulatory action consequently reduces EP300 acetylation levels, thereby suppressing downstream autophagic processes while simultaneously modulating pro-inflammatory responses.

Maltol has been demonstrated to suppress NOD-like receptor protein 3 (NLRP3) inflammasome activation and attenuate the release of pro-inflammatory cytokines interleukin (IL)-1β and IL-18, a phenomenon mediated through its inhibitory effects on ROS generation and caspase-1 enzymatic activity.[35] Maltol promotes nuclear accumulation of transcription factor EB via 5'-adenosine monophosphate-activated protein kinase/mTOR pathway activation to enhance lysosomal function and attenuate autophagy-dependent apoptosis.[36] Nevertheless, the potential existence of a direct regulatory relationship between EP300 and the AMPK/MTOR signaling pathway warrants additional investigation.

Another pharmacologically active compound exerting inhibitory effects on EP300 has been identified as PCA, a primary water-soluble constituent derived from *Salvia miltiorrhiza* Bunge (Lamiaceae), which has been extensively utilized in TCM for cardiovascular therapeutics.[37] Its therapeutic intervention in stroke manifests through multifaceted mechanisms, including autophagy enhancement, anti-inflammatory activity, antioxidant effects, neural regeneration promotion, and apoptosis inhibition. Mechanistically, PCA induces mitophagy activation via the NRF2/PTEN-induced kinase 1/Parkin signaling pathway. Furthermore, experimental evidence demonstrates its capacity to upregulate autophagy-related proteins through modulation of silent information regulator 1 (SIRT1), thereby establishing a dual-pathway regulatory mechanism in autophagic processes.[38] SIRT1 inhibits the transcriptional activation of EP300 by deacetylating EP300 and reducing its acetyltransferase activity.[39] A study showed that MCAO and oxygen-glucose deprivation increased the expression of NLRP3, IL-1β, and NIMA-associated kinase 7, which could be reversed by treatment with PCA.[40] The post-translational acetylation of the EP300 transcriptional coactivator potentiates NLRP3 inflammasome complex assembly and stimulates the secretion of pro-inflammatory cytokines, including IL-1β and IL-18.[41] IL-1β activates the NF-κB and MAPK signaling pathways through its receptor, and activation of these pathways, in turn, promotes EP300 expression and activity.[42] Microglia are immune cells in the central nervous system that are activated during the inflammatory response in stroke. PCA can inhibit inflammation by regulating the polarization state of microglia.[43] EP300 affects the expression of inflammation-related genes and forms immune memory by regulating H3K27ac deposition in microglia. The EP300 inhibitor GNE-049 can inhibit the formation of epigenetic memory and reduce the inflammatory response. It is speculated that PCA may also be a potential EP300 inhibitor.

In TNFα-induced endothelial injury model, PCA inhibits the migration and proliferation of vascular smooth muscle cells and prevents vascular stenosis by inhibiting the NF-κB signaling pathway and reducing the expression of hypoxia-inducible factor 1-alpha (HIF-1α). Meanwhile, TNF-α and other cytokines can mediate the expression of EP300 through NF-κB.[44] Some scholars have confirmed that PCA can improve mitochondrial energy metabolism in the internal capsule after cerebral ischemia/reperfusion, and this mechanism is associated with the inhibition of HIF1α/pyruvate dehydrogenase kinase 1 signaling pathway.[45] Therefore, PCA may inhibit EP300 expression through the NF-κB signaling pathway, thereby reducing HIF-1α acetylation and inhibiting HIF-1α function in the stroke model.

The neuroprotective mechanisms of maltol and PCA in cerebral ischemia appear to involve multiple pathways: (i) Inhibition of EP300-mediated transcriptional regulation;

(ii) activation of autophagic clearance pathways through lysosomal degradation mechanisms; (iii) suppression of aberrant vascular endothelial cell proliferation that predisposes to neovascular stenosis; and (iv) mitigation of neuroinflammatory injury through cytokine modulation in cerebral parenchyma.

Neuroblastoma RAS viral oncogene homolog, a member of the RAS gene family, plays an important role in cell signaling.[46] NRAS mutations are common in melanoma.[47] These mutations are closely related to the occurrence of bone marrow sympathetic neuropathy in hematopoietic system malignancies. Studies have found that NRAS mutations may cause myeloid proliferation and matrix damage by affecting neural signaling in the bone marrow microenvironment, thereby triggering neuropathy.[48]

NRAS gene mutations are also associated with some nervous system diseases, such as neuroblastoma and neurofibromatosis. At present, there is no direct and clear association between NRAS and stroke. However, the N-Ras protein encoded by the *NRAS* gene is part of the RAS/MAPK signaling pathway, which is involved in cell growth, division (proliferation), and differentiation.[49] In the pathogenesis of ischemic stroke, activation of the RAS/MAPK signaling pathway initiates critical pathophysiological cascades. Contemporary research elucidates that p38 MAPK signaling pathway activation demonstrates a significant correlation with neuronal apoptosis in cerebral ischemia-reperfusion injury. Activation of p38 MAPK facilitates neuronal apoptosis; conversely, pharmacological inhibition of this pathway demonstrates neuroprotective potential by attenuating post-stroke neurological deficits. Mechanistic studies reveal that p38 MAPK modulates apoptosis through phosphorylation-dependent regulation of downstream transcription factors and pro-apoptotic protein activation.[50] Emerging evidence indicates that the p38 MAPK signaling pathway mediates cerebral ischemic tolerance induced by cerebral ischemic pre-conditioning through coordinated interactions with downstream regulatory molecules, including peroxisome proliferator-activated receptor γ and NF-κB.[51]

Neuroblastoma RAS viral oncogene homolog promotes cell cycle progression by activating the MAPK signaling pathway. Activation of the MAPK pathway activates extracellular signal-regulated kinase (ERK) 1/2 and p38 MAPK pathways, which promote the activation of microglia (the major immune cells in the brain) and polarize them into proinflammatory M1 type. M1 microglia release a large number of proinflammatory cytokines (such as TNF-α, IL-1β, and IL-6), which aggravate the inflammatory response in brain tissue.[52] The ERK signaling pathway potentiates the production of ROS through the activation of nicotinamide adenine dinucleotide phosphate oxidase and associated signaling cascades, thereby exacerbating the inflammatory cascade via oxidative stress mechanisms.[53] NRAS interacts with PI3K and promotes its activation. Activated PI3K generates phosphatidylinositol triphosphate, which further activates AKT. AKT activation promotes mTOR signaling pathway activation and inhibits autophagy.[54] The constitutive activation of the PI3K–AKT signaling pathway has been implicated in maintaining microglial hyperactivity, driving excessive generation of ROS and proinflammatory cytokines, which collectively potentiate neuroinflammatory pathogenesis through sustained neuroimmune dysregulation.[55]

Albiflorin is a monoterpene glycoside extracted from the roots of the Ranunculaceae plant *Paeonia lactiflora* Pall. Albiflorin significantly improves cell survival rate, reduces intracellular ROS levels, restores mitochondrial transmembrane potential, increases the expression of anti-apoptotic proteins Bcl-2 and Bcl-X, inhibits the expression of the apoptosis marker protein Cleaved Caspase 3, and increases the phosphorylation level of AKT/glycogen synthase kinase 3 beta (GSK-3β).[56] Upon activation, AKT phosphorylates GSK-3β at the Ser9 residue, a post-translational modification that induces conformational alterations in GSK-3β, resulting in its functional inactivation through steric hindrance of the substrate-binding domain.[57] GSK-3β modulates NRAS protein stability through the regulation of leucine zipper-like transcription regulator 1 (LZTR1) activity, a substrate-specific adaptor for the E3 ubiquitin ligase complex that targets Ras family proteins for proteasomal degradation. Mechanistically, LZTR1 facilitates the recruitment of NRAS and other Ras-related proteins to the ubiquitin-proteasome system, thereby controlling their cellular abundance through targeted protein degradation. GSK-3β functions as a negative regulator of the LZTR1–Ras protein interaction. Experimental results demonstrated that pharmacological inhibition of GSK-3β significantly enhanced the binding affinity between LZTR1 and Ras proteins.[58] LZTR1-mediated proteasomal degradation of NRAS was significantly potentiated.

Albiflorin exerts inhibitory effects on oxidative and inflammatory damage *in vivo* through mechanisms involving the suppression of p38 MAPK phosphorylation. This inhibition subsequently attenuates inflammatory responses by mitigating the secretion of pro-inflammatory cytokines and modulating associated signaling pathways.[59] Albiflorin demonstrates the capacity to significantly attenuate malondialdehyde levels while concurrently enhancing superoxide dismutase

enzymatic activity. This phytochemical compound effectively suppresses ROS generation, thereby conferring cytoprotective effects against oxidative stress-induced cellular damage through comprehensive modulation of redox homeostasis.[60] Conversely, albiflorin exerts inhibitory effects on neuroinflammatory responses in post-stroke rat models through modulation of the phosphoglycerate kinase 1 (PGK1)/NRF2/heme oxygenase 1 signaling axis. Mechanistically, albiflorin facilitates PGK1 degradation while inducing sequential activation of the NRF2 signaling cascade, thereby mediating dual therapeutic effects encompassing anti-inflammatory activity and attenuation of oxidative stress-induced pathological progression.[61] NRF2 activation leads to increased expression of antioxidant enzymes that can rapidly remove ROS and prevent further cell damage.[62] The expression of NRF2 downstream genes, such as antioxidant enzymes, alleviates oxidative stress and thus acts as a buffer against excessive activation of the MAPK pathway. This process helps to maintain intracellular redox balance.[63] The suppression of ROS levels induces a significant decrease in oxidative post-translational modulation of NRAS proteins, thereby attenuating their guanosine triphosphate-binding capacity and subsequent enzymatic activity. This biochemical alteration subsequently leads to the inhibition of critical downstream signaling cascades, particularly the MAPK/ERK signaling cascade.[64]

Although limited empirical evidence exists regarding the direct pharmacological effects of albiflorin on NRAS, this compound demonstrates regulatory potential through downstream mediators of the NRAS signaling pathway to mitigate inflammatory and oxidative processes. Notably, albiflorin mechanistically modulates lysine-specific demethylase 1 (LSD1)-mediated microglial activation and ferroptosis pathways, thereby ameliorating neuroinflammatory responses in experimental models.[65] Albiflorin ameliorates cerebral ischemic injury via dual modulation of ferroptosis inhibition and autophagy activation through the PI3K/AKT signaling pathway in MCAO models.[66] Albiflorin exerts inhibitory effects on the c-Jun N-terminal kinase (JNK) signaling pathway, resulting in attenuation of astrocyte activation and pro-inflammatory chemokine secretion.[67] The potential regulatory effects of LSD1 enzymatic activity, the PI3K/AKT signaling pathway, JNK phosphorylation status, and related biochemical mediators on NRAS protein expression levels and post-translational activation mechanisms warrant comprehensive investigation through systematic experimental validation.

In this investigation, NRAS was selected as the molecular target of albiflorin for mechanistic exploration. Our findings suggest that XJDHT's therapeutic efficacy in stroke may be mediated through albiflorin's regulation of the AKT/GSK-3β signaling pathway. Specifically, Albiflorin demonstrates significant inhibitory effects on GSK-3β enzyme activity, thereby reducing NRAS biosynthesis. Furthermore, the compound effectively modulates NRAS–guanosine triphosphate binding dynamics and enzymatic function through ROS level modulation.

5. Conclusion

This study represents the first comprehensive application of multinetwork modeling to investigate the pharmacological mechanism of XJDHT in ischemic stroke treatment, providing novel insights into its therapeutic potential. Systematic network pharmacology analysis identified 24 bioactive components and 424 compound-associated targets relevant to ischemic stroke pathology. Among these, 12 principal active compounds were characterized, including kaempferol, albiflorin, ellagic acid, quercetin, benzoyl paeoniflorin, PCA, paeoniflorin, berberine, pinocembrin, sitosterol, stigmasterol, and 3-hydroxy-2-methyl-4H-pyran-4-one, all of which have been experimentally validated for diverse pharmacological activities. Pathway enrichment analysis revealed that XJDHT's neuroprotective effects are potentially mediated through modulation of MAPK, PI3K–AKT, mTOR, ErbB, and VEGF signaling pathways. Building upon prior research, molecular docking studies identified EP300 and NRAS as critical target proteins, demonstrating that maltol and PCA in XJDHT exert therapeutic effects against ischemic stroke by enhancing autophagy and suppressing inflammatory responses. Furthermore, albiflorin was shown to alleviate neuronal damage through dual mechanisms of anti-inflammatory action and oxidative stress reduction.

These findings elucidate the molecular mechanism underlying XJDHT and establish a theoretical framework for its clinical implementation. However, the current constraints in TCM phytochemical databases necessitate cautious interpretation of network pharmacology predictions, and the incomplete experimental validation of identified targets warrants further investigation. Subsequent research should prioritize comprehensive *in vivo* efficacy assessments of XJDHT in ischemic stroke models, coupled with mechanistic studies to validate the predicted signaling pathways and molecular interactions.

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Conflict of interest

Zhaoyao Chen is the Youth Editorial Board Member of this journal but was not involved in the editorial and peer-review process conducted for this paper, either directly or indirectly. Separately, other authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Writing – review & editing: Zhaoyao Chen

Ethic approval and consent to participate

All animal experiments were conducted in accordance with the guidelines of the Animal Ethics Committee in the Affiliated Hospital of Nanjing University of Traditional Chinese Medicine. Ethical approval was obtained from the Laboratory Animal Ethics Committee, [Hospital of Nanjing University of Traditional Chinese Medicine] (Approval Number: 2023DW-085-01). All procedures followed the "3R principle" (Replacement, Reduction, Refinement).

Consent for publication

Not applicable.

Availability of data

All data supporting the findings of this study are available from the corresponding author upon reasonable request and without restriction.

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ORIGINAL RESEARCH ARTICLE

Comparative visual pathway analysis in competitive sports: A cross-sectional visual evoked potential study

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Abstract

Central visual conduction ability is crucial to human perception and visual skills. In sports, for instance, it enables athletes to gather information, predict movements, make quick decisions, and perform precise motor actions. In the present study, modern electrophysiological tools, such as visual evoked potentials (VEPs), were employed to assess central visual transmission. The goal was to analyze three VEP waveforms (N75, P100, and N145) in male athletes (n = 100) from cricket, yogasana, karate, and gymnastics, together with a control group of physically active boys without specific training in these disciplines. For the P100 waveform, yogasana and karateka practitioners showed significantly shorter latencies compared to all other groups (p<0.05), followed by cricketers, who had latencies similar to gymnasts. The N75 waveform for yogasana and karate practitioners showed significantly faster transmission times than the cricket and control groups (p<0.05). Furthermore, the N75 waveform for the left eye in gymnasts had shorter latency compared to cricketers and controls. The N145 wave for the right eye in yogasana and karate practitioners had shorter latencies compared to those of other athletes (p<0.05). Meanwhile, karatekas showed higher amplitudes than those practicing yogasana and the controls, indicating that training experience might be a key factor. This study provides valuable insights into the unique visual processing demands and capabilities of athletes in various disciplines. Yogasana and karate, being gaze-oriented practices that integrate mind–body control, showed shorter latency across all groups, followed by ocularly dominant cricketers, visually trained gymnasts, and controls.

Keywords: Central visual transmission; Sports players; Visual processing demands; Gaze-oriented activity

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1. Introduction

Central visual conduction ability plays a pivotal role in human perception, particularly in athletes whose performance depends significantly on perception, interpretation, and processing of visual stimuli.[1-3] Visual skills enable athletes to gather essential information from their environment, predict movements, make quick decisions, and perform precise motor actions during both training and competition.[4] Notably, specific sports require

specific visual skills, which can vary significantly depending on the nature of the sports. For example, cricket, as a popular ball game, requires players to have quicker visual reaction time for better hitting and fielding performance.[5] In contrast, individual emerging sports, such as yogasana (yoga postures), underscore the importance of visual skills for maintaining mind–body balance.[6] Visual skills are also crucial for karatekas for sensing opponents and identifying potential threats, along with an instinctive ability to detect hidden dangers.[7] For the successful execution of gymnastic skills, gymnasts need to possess specific visual acuity, coordination, and visualization abilities.[8]

Traditionally, visual reaction times were denoted as a measure to understand quickness and the connection between the muscular system and the higher centers of the brain in the visual pathway, the occipital lobe.[9-11] As technology advanced, evoked potentials (EPs), a non-invasive electrophysiological measurement, emerged and deepened our understanding of the neural mechanisms of central visual processing and its connection to sensory stimuli. Specifically, visual EPs (VEPs) are used to assess the central visual pathway.[12] In general, three standard stimulus protocols are defined for VEP recording,[13] of which the flash VEP protocol was utilized in this comparative study. The waveforms of flash VEP consist of three distinct phases: An initial negative deflection (N75), followed by a prominent positive deflection (P100), and a later negative deflection (N145).[14] Together, these phases form the negative–positive–negative (NPN) complex, and its measurements are interpreted as peak latency and peak-to-peak amplitudes.[15] This VEP complex measures the conduction time from the retina to the occipital cortex through the visual optic pathway using a mid-occipital electrode. In the NPN complex, the P100 peak latency is the most pronounced and stable waveform.[16] It produces little variation in latency compared to the waveforms from lateral electrodes and has a steady amplitude along with minimal intra- and inter-variability.[17] Previous studies have shown differences in VEP waveforms between athletes and non-athletes, indicating variations in visuo-occipital functioning.[11,18-22]

Considering traditional models of attention and modern VEP measures, the present study aimed to measure and analyze VEPs across athletes from cricket, yogasana, karate, and gymnastics to explore differences in visual processing. While previous research has explored VEPs in specific sports, a direct comparison across mind–body activities, team sports, and individual sports is lacking. This study fills this gap by analyzing how distinct training methods shape neural conduction and visual processing. It also highlights sport-specific neural adaptations, the contrast between dynamic and internal focus-driven processing, and the need for long-term assessments of training effects. These insights can help refine targeted training strategies and optimize athletic performance.

2. Methods

2.1. Participants

The present cross-sectional study was conducted among the male school-going population aged 12 – 18 years, with a mean age of 15.8 ± 1.89 years, from various regions of West Bengal, India. The participants included practitioners of competitive sports, such as yogasana, karate, cricket, and gymnastics, all of whom had a minimum of state-level participation and were engaged in regular practice. Their training age ranged from 6 to 8 years, with a mean training age of 6.16 ± 1.78 years. While yogasana, karate, and gymnastics are considered art forms, and cricket is a team sport, all are currently represented as sports. All subjects were held at least state-level positions, justifying their practical applicability. On the other hand, the control group comprised healthy school-going boys who were free from disease, performed daily activities regularly, and engaged in active play without specific skill training, particularly in yogasana, karate, cricket, or gymnastics. This control group regularly participated in physical activities, such as individual or team games, for similar durations per week as the practitioner groups.

Subjects with ophthalmic disparities and reduced visual acuity, females, and the sedentary population were excluded from the study. Diet, nutrition, and socioeconomic status were not considered criteria for inclusion or exclusion.

2.2. Study design

G*Power version 3.1.9.4 for Windows (2020) (University of Düsseldorf, Germany)[23] was used to calculate a priori sample size and determined that a total of 100 participants was sufficient, based on an error probability of 0.05, power of 0.70, and an effect size of 0.32. Based on this calculation, a subject pool of 118 individuals was formulated, and a convenience sample of 100 subjects was selected from the pool. The 100 subjects were categorized into five groups: The yogasana group, karate group, cricket group, gymnastics group, and control group, with 20 subjects in each group. Figure 1 presents the flow diagram of the present study. The participants, along with their parents and trainers from their respective authorized training organizations and school authorities, attended three pre-data collection meetings and provided informed consent to participate in the study. They were guided throughout the process, and the required assent and consent forms were duly signed at the start of data collection. The research

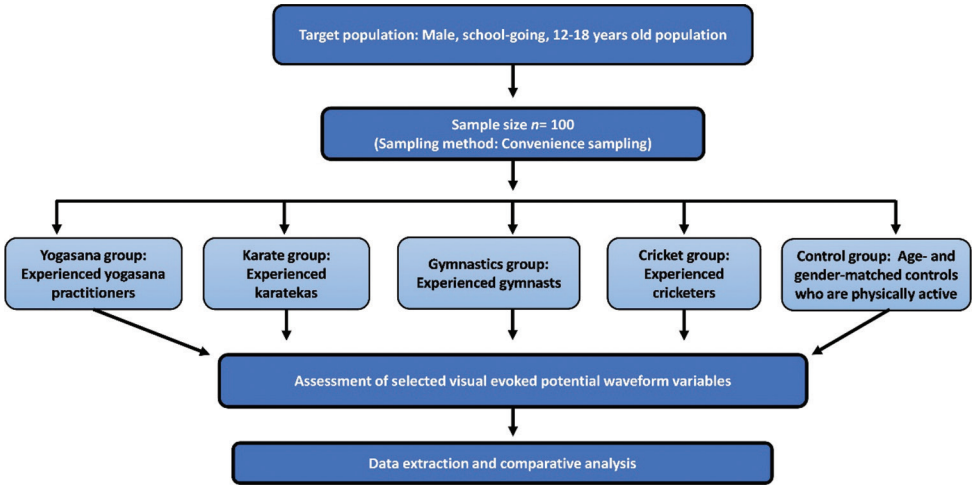


Figure 1. Study design of the cross-sectional experimentation

protocol and design were approved by the Institutional Ethics Committee for Human Research at the university where the study was performed (Approval No.: VB/IECHR/2021/9), and the study adhered to the National Ethical Guidelines for Biomedical and Health Research involving Human Participants of the Indian Council of Medical Research (ICMR).[24,25] The study was also approved by the Clinical Trial Registry of India (CTRI; Registration No.: CTRI/2021/06/034483). The research was conducted in accordance with the Helsinki Declaration.

2.3. Visual sensory variable and data collection process

The visual sensory variable comprises VEP, measured using a goggle-based flash VEP system containing red light-emitting diodes (LEDs). The compact goggles provide a large field of stimulation, minimizing the fluttering of gaze. The RMS Salus 4C (Recorders and Medicare Systems Private Limited, India) EP recorder was used. After cleansing and degreasing the scalp with alcohol, standard disc electroencephalogram (EEG) electrodes were placed based on the 10 – 20 international system of EEG placements.[13,26] For VEP, the standard method recommended by the International Federation of Clinical Neurophysiology and the International Society for Clinical Electro-Physiology of Vision (ISCEV)[26] was followed. The VEP protocol is confined to a three-channel montage, with active electrodes positioned at the midline and two lateral occipital sites. The sensitivity was set at 1 – 100 Hz while the impedance was kept below 5 kΩ. The quality of measurement was ensured by maintaining the reliability of the equipment and through critical observations by technical experts. The testers, who were part of the research core team, were well-informed and qualified to conduct data collection. They possessed full awareness of the validation protocols and demonstrated technical responsiveness in operating the equipment. Moreover, they were assisted by experienced neurocognitive experts. Good clinical practices were adhered to during the data collection procedures. Participants were instructed to lie calmly in a quiet, sound-controlled room before data collection. Monocular recordings were conducted separately for the left and right eyes, and electrode impedance was checked after each trial. These procedures were implemented in accordance with the ICMR guidelines.[24,25]

2.4. Psychometrics and design

EP recordings are highly subtle and sensitive neurophysiological measurements. Therefore, a critical check on technical specifications was conducted based on the RMS Salus 4C manual, expert opinions, and previous research references. The electrode impedance was calibrated daily and maintained below 5 kΩ to minimize artifacts and noise before data collection. Inter-electrode impedance was kept below 2 kΩ and checked at each subject change. For VEP recordings, data filtration took place within the range of 1 – 100 Hz, and LED luminance was maintained at 3 cd·s/m^2. All other detailed specifications are published in previous works.[22]

2.5. Statistical analysis

Descriptive statistics were reported as mean ± SD, with a significant level of 0.05 (p<0.05) following a normality check using the Kolmogorov–Smirnov test. Group comparisons were conducted using one-way analysis of variance (ANOVA), and all statistical analyses were performed using the Statistical Package for the Social Sciences 20.0 software for Windows (IBM, USA). When the ANOVA results were significant, the least significant difference (LSD) *post hoc* test was conducted to determine

which specific group means differed significantly, allowing for assessment of intergroup significance.

3. Results

The demographic features of the participants, including age and body mass index (BMI), did not significantly differ among the five groups. The mean age of the practitioner group was 15 ± 0.40 years, whereas that of the controls was 15.4 ± 1.79 years. The BMI was consistent across groups, with the mean BMI being 19.87 ± 3.47 (yogasana), 19.14 ± 2.24 (karate), 20.22 ± 2.98 (cricket), 21.19 ± 2.01 (gymnastics), and 21.12 ± 2.09 kg/m^2 (controls).

For the VEP (Figure 2), specifically the P100 waveform, the yogasana practitioners (left eye: 94.47 ± 2.43 ms; right eye: 94.53 ± 2.54 ms) and karatekas (left eye: 93.60 ± 1.50 ms; right eye: 93.69 ± 3.32 ms) demonstrated significantly shorter waveform latencies than the gymnasts (left eye: 101.50 ± 6.62 ms; right eye: 103.41 ± 7.33 ms), cricketers (left eye: 97.00 ± 3.01 ms; right eye: 97.01 ± 2.84 ms), and controls (left eye: 105.07 ± 2.23 ms; right eye: 105.63 ± 3.21 ms). In addition, the cricketers recorded significantly shorter P100 latencies than the gymnasts and controls. Notably, the gymnasts showed significantly shorter P100 latency than the controls only in the left eye.

For the N75 waveform, yogasana practitioners (left eye: 61.36 ± 2.56 ms; right eye: 62.02 ± 3.67 ms) and karatekas (left eye: 62.19 ± 2.41 ms; right eye: 63.15 ± 2.84 ms) showed significantly shorter transmission time than the cricketers (left eye: 67.41 ± 7.02 ms; right eye: 67.22 ± 7.18 ms) and controls (left eye: 65.86 ± 5.46 ms; right eye: 66.29 ± 7.13 ms). Notably, in comparison to the cricketers and controls, the gymnasts showed a markedly shorter N75 latency only in the left eye (left eye: 63.27 ± 3.76 ms).

For the N145 waveform, the yogasana practitioners (left eye: 156.4 ± 9.65 ms; right eye: 153.83 ± 10.01 ms) and karatekas (left eye: 156.56 ± 10.62 ms; right eye: 154.48 ± 14.03 ms) showed markedly shorter latencies compared to the controls (left eye: 162.53 ± 12.23 ms; right eye: 162.39 ± 10.77 ms); however, statistically significance were only seen in the right eye for both the groups.

The karatekas (3.91 ± 2.56 μV) showed significantly lower P100 – N75 amplitude compared to the yogasana practitioners (5.79 ± 3.91 μV) and controls (5.64 ± 2.72 μV) (Figure 3). Table 1 presents all the variables with their descriptive statistics and one-way ANOVA parameters, whereas Table 2 presents the variables with inter-group comparisons using the LSD *post hoc* test.

4. Discussion

In a nutshell, the results revealed that among all groups, the karatekas exhibited the shortest P100 latencies, indicating the fastest visual processing times. The yogasana practitioners followed closely, also showing significantly shorter latencies compared to the cricketers, gymnasts,

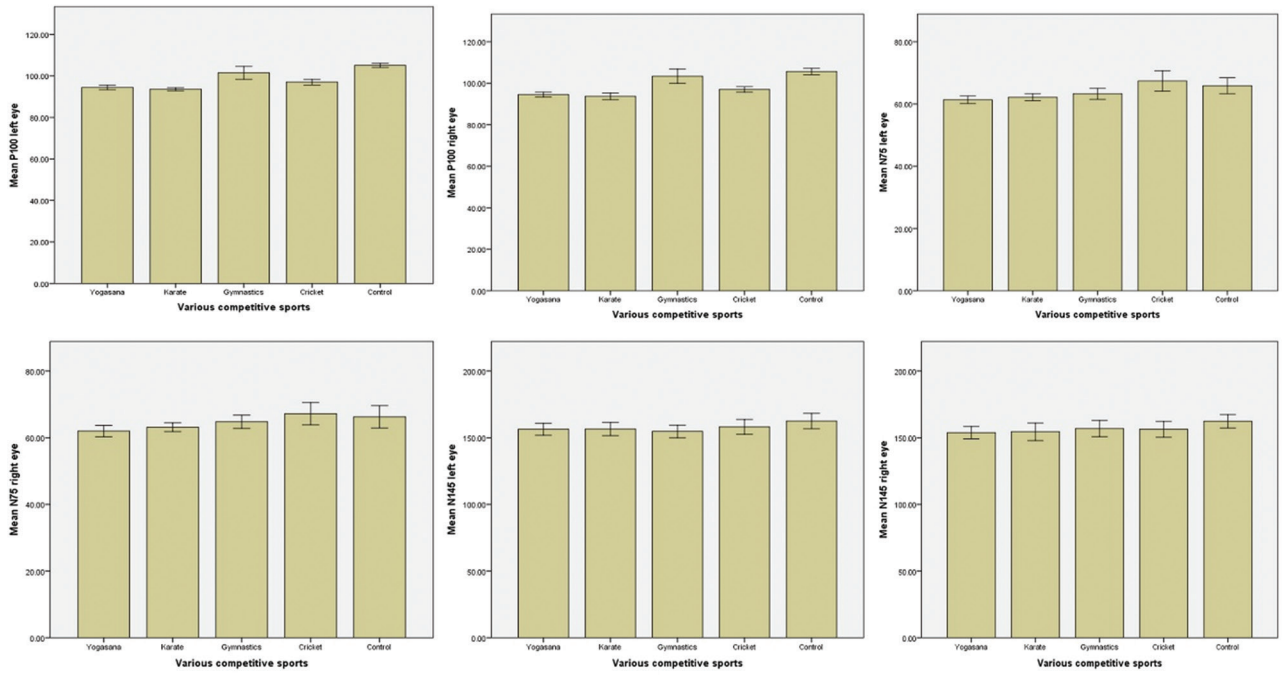


Figure 2. Visual evoked potential latencies of the negative–positive–negative complex across the five groups

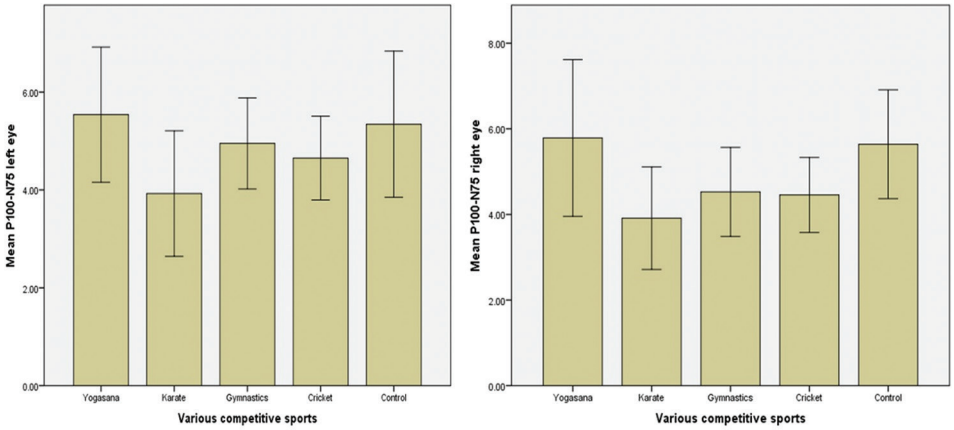


Figure 3. Visual evoked potential amplitude across the five groups

and controls. The cricketers, while not as rapid as the karatekas or yogasana practitioners, demonstrated faster visual transmission times than the gymnasts and controls. In contrast, the gymnasts and controls demonstrated the longest latencies, indicating slower visual processing speeds. These findings highlight the positive impacts of specific physical activities, particularly martial arts and yogasana, on enhancing visual response times.

4.1. Visuo-motor neural response mechanism and its variations among sports players

Advancements in technology have introduced improved methods of measurement, such as VEP recording. The P100 component of VEPs reflects the brain's response to visual stimuli, providing insights into the speed of information processing and selective attention. VEPs measure the electrical signals elicited by visual stimulation, with responses recorded from the visual cortex in the occipital lobes. The anterior visual pathway signaling was quantified through the flash VEP setup, based on ISCEV.[13,26] Latency, measured in milliseconds, indicates the time taken for a visual stimulus to travel from the retina to the occipital cortex, and is typically represented through the NPN complex waveform. Although less effective for detecting training effects, amplitude was also recorded in this study.[12] This study focuses on visual evoked responses to assess the functional integrity of the visual pathway from the retina, via the optic nerves, to the visual cortex. Compared to scanning techniques, such as magnetic resonance imaging (MRI), VEPs provide a more quantifiable measure of the functional integrity of the optic pathways.[27]

The most common approach for assessing the visual-sensory–motor abilities relevant to sports performance involves comparing athletes from different sports with non-athletes, a method employed in the present study. Various mechanisms have been proposed by previous researchers, some of which are discussed here.

Athletes may possess inherently superior visual-system physiology, termed "visual hardware," which allows for more efficient reception of visual information. Alternatively, they may develop enhanced perceptual–cognitive abilities, or "visual software," through practice involving visual information processing.[28,29] Neural responses to visual stimuli consist of bottom-up and top-down processes. Bottom-up mechanisms involve initial visual information processing, beginning with light entering the eye, stimulating the photoreceptors, and transmitting information through the optic nerve to the brain, leading to motor output. In contrast, top-down mechanisms involve the brain's processing of visual information, incorporating previous experiences from training, competition, or visual skills to influence the interpretation of new information and determine appropriate responses.[30,31] These mechanisms prioritize specific regions or objects within the visual scene for processing while filtering out irrelevant information.

Attention can be driven by both endogenous factors (intrinsically generated goals and expectations) and exogenous factors (stimulus-driven cues).[32] In addition, the magnocellular pathway, a key component of the visual system responsible for motion detection and processing low spatial frequencies, may be enhanced through regular sports training. This enhancement can lead to sharper visual perception, faster reaction times, and improved motor coordination, ultimately refining an athlete's ability to track moving objects and anticipate motion dynamics more effectively.[33,34]

4.2. Sports training and vision among athletes from different sports

Sports training aims to enhance perceptual mechanisms by improving the visual skills necessary for optimal sports

Table 1. Descriptive statistics of the visual evoked potential tests across five groups

Variables	Groups	*n*	Mean±standard deviation	ANOVA groups	Sum of squares	df	Mean square	F-ratio	*p*-value
P100 latency, left eye (ms)	Yogasana	20	94.47±2.43	Between groups	1890.548	4	472.637	35.842	<0.001***
	Karate	20	93.60±1.50	Within groups	1252.738	95	13.187		
	Gymnastics	20	101.50±6.62	Total	3143.286	99			
	Cricket	20	97.00±3.01						
	Control	20	105.07±2.23						
	Total	100	98.33±5.63						
P100 latency, right eye (ms)	Yogasana	20	94.53±2.54	Between groups	2308.984	4	577.246	32.190	<0.001***
	Karate	20	93.69±3.32	Within groups	1703.584	95	17.932		
	Gymnastics	20	103.41±7.33	Total	4012.567	99			
	Cricket	20	97.01±2.84						
	Control	20	105.63±3.21						
	Total	100	98.85±6.37						
N75 latency, left eye (ms)	Yogasana	20	61.36±2.56	Between groups	517.264	4	129.316	6.121	<0.001***
	Karate	20	62.19±2.41	Within groups	2007.068	95	21.127		
	Gymnastics	20	63.27±3.76	Total	2524.331	99			
	Cricket	20	67.41±7.02						
	Control	20	65.86±5.46						
	Total	100	64.02±5.05						
N75 latency, right eye (ms)	Yogasana	20	62.02±3.67	Between groups	369.987	4	92.497	3.264	0.015*
	Karate	20	63.15±2.84	Within groups	2691.88	95	28.336		
	Gymnastics	20	64.80±4.21	Total	3061.868	99			
	Cricket	20	67.22±7.18						
	Control	20	66.29±7.13						
	Total	100	64.69±5.56						
N145 latency, left eye (ms)	Yogasana	20	156.40±9.65	Between groups	705.363	4	176.341	1.457	0.222
	Karate	20	156.56±10.62	Within groups	11501.31	95	121.066		
	Gymnastics	20	154.73±10.18	Total	12206.673	99			
	Cricket	20	158.11±11.99						
	Control	20	162.53±12.33						
	Total	100	157.66±11.10						
N145 latency, right eye (ms)	Yogasana	20	153.83±10.01	Between groups	913.813	4	228.453	1.539	0.197
	Karate	20	154.48±14.03	Within groups	14105.33	95	148.477		
	Gymnastics	20	156.93±13.02	Total	15019.143	99			
	Cricket	20	156.36±12.65						
	Control	20	162.39±10.77						
	Total	100	156.80±12.32						
P100–N75 amplitude, left eye (μV)	Yogasana	20	5.54±2.95	Between groups	32.284	4	8.071	1.197	0.317
	Karate	20	3.93±2.74	Within groups	640.614	95	6.743		
	Gymnastics	20	4.95±1.99	Total	672.899	99			
	Cricket	20	4.65±1.83						
	Control	20	5.34±3.19						
	Total	100	4.88±2.61						

(*Cont'd....*)

Table 1. (*Continued*)

Variables	Groups	*n*	Mean±standard deviation	ANOVA groups	Sum of squares	df	Mean square	F-ratio	*p*-value
P100–N75 amplitude, right eye (μV)	Yogasana	20	5.79±3.91	Between groups	52.773	4	13.193	1.751	0.145
	Karate	20	3.91±2.56	Within groups	715.714	95	7.534		
	Gymnastics	20	4.53±2.22	Total	768.487	99			
	Cricket	20	4.45±1.87						
	Control	20	5.64±2.72						
	Total	100	4.86±2.79						

Notes: Statistical analysis conducted using one-way ANOVA; **p*<0.05, ***p*<0.01, ****p*<0.001.
Abbreviation: ANOVA: Analysis of variance.

Table 2. Pairwise comparison of the visual evoked potential variables across five groups

Groups	*n*	Left eye				Right eye			
		Mean±SD	Mean difference	Standard error difference	*p*-value	Mean±SD	Mean difference	Standard error difference	*p*-value
P100 latency (ms)									
Yogasana	20	94.47±2.43	0.87000	1.14833	0.451	94.53±2.54	0.84000	1.33912	0.532
Karate	20	93.60±1.50				93.69±3.32			
Yogasana	20	94.47±2.43	7.03150	1.14833	<0.001***	94.53±2.54	8.88150	1.33912	<0.001***
Gymnastics	20	101.50±6.62				103.41±7.33			
Yogasana	20	94.47±2.43	2.53000	1.14833	0.030*	94.53±2.54	2.48000	1.33912	0.067
Cricket	20	97.00±3.01				97.01±2.84			
Yogasana	20	94.47±2.43	10.60000	1.14833	<0.001***	94.53±2.54	11.10000	1.33912	<0.001***
Control	20	105.07±2.23				105.63±3.21			
Karate	20	93.60±1.50	7.90150	1.14833	<0.001***	93.69±3.32	9.72150	1.33912	<0.001***
Gymnastics	20	101.50±6.62				103.41±7.33			
Karate	20	93.60±1.50	3.40000	1.14833	0.004**	93.69±3.32	3.32000	1.33912	0.015*
Cricket	20	97.00±3.01				97.01±2.84			
Karate	20	93.60±1.50	11.47000	1.14833	<0.001***	93.69±3.32	11.94000	1.33912	<0.001***
Control	20	105.07±2.23				105.63±3.21			
Gymnastics	20	101.50±6.62	4.50150	1.14833	<0.001***	103.41±7.33	6.40150	1.33912	<0.001***
Cricket	20	97.00±3.01				97.01±2.84			
Gymnastics	20	101.5±6.62	3.56850	1.14833	0.002**	103.41±7.33	2.21850	1.33912	0.101
Control	20	105.07±2.23				105.63±3.21			
Cricket	20	97.00±3.01	8.07000	1.14833	<0.001***	97.01±2.84	8.62000	1.33912	<0.001***
Control	20	105.07±2.23				105.63±3.21			
N75 latency (ms)									
Yogasana	20	61.36±2.56	0.83000	1.45351	0.569	62.02±3.67	1.13500	1.68332	0.502
Karate	20	62.19±2.41				63.15±2.84			
Yogasana	20	61.36±2.56	1.91100	1.45351	0.192	62.02±3.67	2.78150	1.68332	0.102
Gymnastics	20	63.27±3.76				64.80±4.21			
Yogasana	20	61.36±2.56	6.05000	1.45351	<0.001***	62.02±3.67	5.20500	1.68332	0.003**
Cricket	20	67.41±7.02				67.22±7.18			
Yogasana	20	61.36±2.56	4.50000	1.45351	0.003**	62.02±3.67	4.27500	1.68332	0.013*
Control	20	65.86±5.46				66.29±7.13			
Karate	20	62.19±2.41	1.08100	1.45351	0.459	63.15±2.84	1.64650	1.68332	0.330
Gymnastics	20	63.27±3.76				64.80±4.21			
Karate	20	62.19±2.41	5.22000	1.45351	0.001**	63.15±2.84	4.07000	1.68332	0.018*
Cricket	20	67.41±7.02				67.22±7.18			

(*Cont'd....*)

Table 2. (*Continued*)

Groups	*n*	Left eye				Right eye			
		Mean±SD	Mean difference	Standard error difference	*p*-value	Mean±SD	Mean difference	Standard error difference	*p*-value
Karate	20	62.19±2.41	3.67000	1.45351	0.013*	63.15±2.84	3.14000	1.68332	0.065
Control	20	65.86±5.46				66.29±7.13			
Gymnastics	20	63.27±3.76	4.13900	1.45351	0.005**	64.80±4.21	2.42350	1.68332	0.153
Cricket	20	67.41±7.02				67.22±7.18			
Gymnastics	20	63.27±3.76	2.58900	1.45351	0.078	64.80±4.21	1.49350	1.68332	0.377
Control	20	65.86±5.46				66.29±7.13			
Cricket	20	67.41±7.02	1.55000	1.45351	0.289	67.22±7.18	0.93000	1.68332	0.582
Control	20	65.86±5.46				66.29±7.13			
N145 latency (ms)									
Yogasana	20	156.40±9.65	0.15850	3.47946	0.964	153.83±10.01	0.65500	3.85327	0.865
Karate	20	156.56±10.62				154.48±14.03			
Yogasana	20	156.4±9.65	1.67200	3.47946	0.632	153.83±10.01	3.10150	3.85327	0.423
Gymnastics	20	154.73±10.18				156.93±13.02			
Yogasana	20	156.40±9.65	1.71000	3.47946	0.624	153.83±10.01	2.53500	3.85327	0.512
Cricket	20	158.11±11.99				156.36±12.65			
Yogasana	20	156.40±9.65	6.12500	3.47946	0.082	153.83±10.01	8.56500	3.85327	0.029*
Control	20	162.53±12.33				162.39±10.77			
Karate	20	156.56±10.62	1.83050	3.47946	0.600	154.48±14.03	2.44650	3.85327	0.527
Gymnastics	20	154.73±10.18				156.93±13.02			
Karate	20	156.56±10.62	1.55150	3.47946	0.657	154.48±14.03	1.88000	3.85327	0.627
Cricket	20	158.11±11.99				156.36±12.65			
Karate	20	156.56±10.62	5.96650	3.47946	0.090	154.48±14.03	7.91000	3.85327	0.043*
Control	20	162.53±12.33				162.39±10.77			
Gymnastics	20	154.73±10.18	3.38200	3.47946	0.334	156.93±13.02	0.56650	3.85327	0.883
Cricket	20	158.11±11.99				156.36±12.65			
Gymnastics	20	154.73±10.18	7.79700	3.47946	0.027*	156.93±13.02	5.46350	3.85327	0.159
Control	20	162.53±12.33				162.39±10.77			
Cricket	20	158.11±11.99	4.41500	3.47946	0.208	156.36±12.65	6.03000	3.85327	0.121
Control	20	162.53±12.33				162.39±10.77			
P100–N75 amplitude (μV)									
Yogasana	20	5.54±2.95	1.61150	0.82118	0.053	5.79±3.91	1.87400	0.86798	0.033*
Karate	20	3.93±2.74				3.91±2.56			
Yogasana	20	5.54±2.95	0.58850	0.82118	0.475	5.79±3.91	1.25900	0.86798	0.150
Gymnastics	20	4.95±1.99				4.53±2.22			
Yogasana	20	5.54±2.95	0.88700	0.82118	0.283	5.79±3.91	1.33100	0.86798	0.128
Cricket	20	4.65±1.83				4.45±1.87			
Yogasana	20	5.54±2.95	0.19450	0.82118	0.813	5.79±3.91	0.14650	0.86798	0.866
Control	20	5.34±3.19				5.64±2.72			
Karate	20	3.93±2.74	1.02300	0.82118	0.216	3.91±2.56	0.61500	0.86798	0.480
Gymnastics	20	4.95±1.99				4.53±2.22			
Karate	20	3.93±2.74	0.72450	0.82118	0.380	3.91±2.56	0.54300	0.86798	0.533
Cricket	20	4.65±1.83				4.45±1.87			
Karate	20	3.93±2.74	1.41700	0.82118	0.088	3.91±2.56	1.72750	0.86798	0.049*
Control	20	5.34±3.19				5.64±2.72			
Gymnastics	20	4.95±1.99	0.29850	0.82118	0.717	4.53±2.22	0.07200	0.86798	0.934
Cricket	20	4.65±1.83				4.45±1.87			
Gymnastics	20	4.95±1.99	0.39400	0.82118	0.632	4.53±2.22	1.11250	0.86798	0.203
Control	20	5.34±3.19				5.64±2.72			
Cricket	20	4.65±1.83	0.69250	0.82118	0.401	4.45±1.87	1.18450	0.86798	0.176
Control	20	5.34±3.19				5.64±2.72			

Notes: Statistical analysis conducted using the least significant difference *post hoc* test; *$p<0.05$, **$p<0.01$, ***$p<0.001$.
Abbreviation: SD: Standard deviation.

performance. The primary goal of any sports training program is to help athletes process larger quantities of information in a shorter time, while priming the perceptual and effector mechanisms for subsequent information.[35] This process ultimately improves the speed and efficiency of the decision-making process, which is further enhanced by feedback on visual attention and the use of mental imagery techniques.[36]

Training methods that simulate sports-related conditions provide additional benefits by facilitating the transfer of perceptual and cognitive improvements to real performance settings. Figure 4 describes the processing of various feedback based on perceptual, decision-making, and effector mechanisms, each of which varies depending on the sport. Focusing specifically on individual sports, the results of the present study provide a clearer picture regarding the visual sensory reception and central cognitive processing mechanisms of the brain.

First, considering yogasana and karate, the yogasana practitioners demonstrated the shortest P100 latencies – the most reproducible component of the VEP – for both the left and right eyes compared to other sports, apart from karate, which showed similar results. These results align with previous studies on yogasana, karate,[37-41] and other mind–body activities.[42] A possible explanation lies in the neural processing of visual stimuli, wherein signals travel from the retina through the lateral geniculate nucleus to the primary visual cortex, and their divergence into two neural pathways that provide additional signal processing: the dorsal pathway, which provides information about spatial properties; and the ventral pathway, which provides further information about object detailing and identification, through the inferior temporal cortex. These pathways converge in the areas of the prefrontal cortex and posterior parietal cortex, which are both key areas for decision-making and perceptual integration, offering advantages in these sports.[43,44] In addition, one common factor in both yogasana and karate is the use of *drishti* (gaze), a focal point to deepen the primary movement of the yogic pose and to keep away the mind from wandering so that it is tamed and engaged inward. *Drishti* is a natural eye movement directed by the alignment of the body in a given posture.[45] Previous research on *drishti*, scientifically known as focal point concentration of gazing, has demonstrated improvements in neural processing and enhanced visual cognition through yogic activity.[46,47]

Similarly, karate training involves various components, including *kihon*, *kata*, and *kumite*, with *kihon* being the basics of karate – e.g., punches, blocks, kicks, and stances. *Kata* involves the performance of predefined sequences of offensive and defensive movements in a standardized order, while *kumite* refers to free fighting or sparring. In all these practices, movements of the eye are highly pertinent. Direct gaze shifts toward a visual target are known as overt attention, and the ability to attend to peripheral targets without shifting gaze is known as covert attention. These visual attention mechanisms, combined with neural conduction to attain optimum transient and spatial attention, are the prime neurocognitive requirements in martial arts, such as karate.[22] Other studies on martial meditative art forms, such as *qigong*, have shown similar results.[42] Over time, the development of such techniques, along with mental rehearsal through mental imagery, enhances the ability to accurately locate and interpret advanced visual cues. This offers significant potential advantages in real-life situations for karatekas and supports meditative indications for yogasana practitioners.

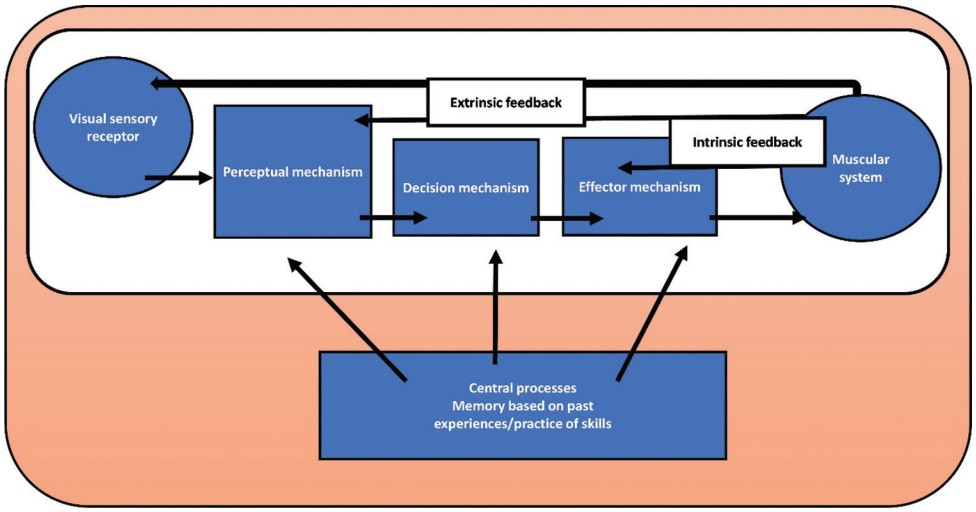


Figure 4. Sports performance training and its visual sensory mechanism

The next group exhibiting shorter visual transmission times was the cricket group. The underlying reasons could be ocular dominance – the preference for visual input from one eye over the other, similar to handedness. Previous studies have suggested similar reasons.[48,49] While handedness typically aligns with the dominant cerebral hemisphere, ocular dominance does not necessarily follow the same pattern, as each eye projects to both cerebral hemispheres. Nevertheless, previous research has demonstrated the effects of handedness on cognition in the general population and athletes such as baseball players.[50,51] A short visual transmission time is crucial in high-speed sports, such as cricket, where athletes must coordinate their eyes, hands, and body.[5,11] Notable cross-dominant athletes are recognized for their exceptional hand–eye coordination. It is possible that consistent exposure to sport-specific demands contributes to improved visual transmission latency. Similar findings have been reported in table tennis players, where enhanced neural efficiency along visual pathways was observed using functional MRI, and more recently in skeet shooters, where rapid visual processing was also evident.[52,53]

Gymnastics, as an individual sport, requires athletes to develop specific visual acuity, coordination, and visualization skills to perform intricate movements with precision and elegance. It relies less on immediate visual feedback from teammates or opponents, instead emphasizing skills such as visual acuity, field of vision, and visual imagery – skills essential for executing movements gracefully and with balance.[8] As a result, the frontal cortex may be less involved in visual object discrimination in gymnasts.[54] This could be a reason for this group to exhibit longer P100 latencies compared to other athletes in this study.

Previous VEP analyses have revealed a relationship between training experience and the N75–P100 amplitude. Specifically, more experienced athletes were characterized by a lower amplitude of this parameter, regardless of the type of stimulation and the viewing conditions during the tests. In the present study, the lowest amplitude was observed in the karatekas, who were the most experienced group among all other sports groups. Conversely, other findings suggest that spatial stimuli, when visually activated and trained accordingly, could lead to heightened N75–P100 amplitude.[55] This may explain the higher amplitude observed in the yogasana group in the present study, as such yogic practices often involve spatial awareness for improved body alignment and posture, as well as a reduced risk of injury.

Research has shown that the traditional theory of sensory processing demonstrates how visual stimuli are transmitted from muscles to the brain's processing lobes, supporting memory and attention development. In addition, sports training has been found to enhance the speed of visually stimulated responses compared to non-athletes or active, age- and gender-matched controls. Recent studies have highlighted the use of various sports-visual-training techniques to further improve visual latency, ultimately enhancing on-field or on-court performance.[53,56] Emerging evidence has also shown how sports visual training is being used to enhance motor efficiency and performance.[56,57]

While previous studies have compared VEP measures between athletes and non-athletes, or between one or two sports and controls, the present study expands this scope by examining VEP measurements across four practitioner groups. The aim was to understand the mechanisms underlying visual stimulus transmission and to compare these with active, gender-matched controls. Despite certain limitations, the study offers valuable insights into the relationship among VEP measures, visual perception mechanisms, types of sport, training modalities, training experience, and training age. The findings indicate that mental imagery and gaze skills, most practiced by yogasana practitioners and karatekas, are associated with the fastest visual transmission. Cricketers exhibited the next fastest response time, followed by gymnasts, who still outperformed the controls.

The study excluded sedentary children and female participants, with the latter omitted due to post-pandemic disruptions in their respective training regimens, which could be a limitation. Furthermore, post-pandemic parental concerns limited female participation in laboratory tests more than male participation, contributing to their exclusion. To address socioeconomic and nutritional variability, all participating schools were chosen from similar socioeconomic backgrounds in West Bengal, and all students received mid-day meals, ensuring a degree of homogeneity. However, certain factors such as diet, nutritional status, socioeconomic status, and sleep profiles were not directly controlled. Despite this, the study maintained highly specific inclusion criteria, minimizing potential confounding effects across the selected groups. As a cross-sectional study, certain limitations exist. Future research should consider longitudinal or intervention-based designs, while also accounting for potential sources of measurement error (e.g., VEP waveform variability and electrode impedance fluctuations).

5. Conclusion

Different sports demand specific visual skills, such as shorter reaction time in cricket, mind–body balance in yogasana, opponent sensing in karate, and visual acuity in gymnastics. The variability in bioelectrical function along

the visual pathway – across central and peripheral locations under monocular and binocular conditions – is interrelated with the perceptual requirements specific to each sport. This functional variability is more pronounced in athletes with more training experience and mind–body activities than those in other team-based or individual sports.

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Conflict of interest

The authors declare they have no competing interests.

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Writing – review & editing: Puneet Bhattacharya, Sridip Chatterjee

Ethics approval and consent to participate

The research protocol and design were approved by the Institutional Ethics Committee for Human Research at the university where the study was performed (Approval No.: VB/IECHR/2021/9). The study was approved by the Clinical Trial Registry of India (Regn. No: CTRI/2021/06/034483). The research was conducted in accordance with the Helsinki Declaration. The written consent to participate form was acquired from the participants at the start of data collection.

Consent for publication

Complete written and verbal informed consent was obtained from the subjects and parents for the publication of this study.

Availability of data

Not applicable.

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

Imbalance of gut *Bacteroides* and *Prevotella* contributes to memory decline in ovariectomized mice fed with high-fat diet

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Abstract

The rates of incidence and progression of Alzheimer's disease (AD) vary significantly among post-menopausal women. However, the mechanisms that underlie this phenomenon remain obscure. Here, we established the high-fat diet-fed ovariectomized (OVXHF) mouse model and observed significant memory deficits in this model. Using 16S rDNA sequencing, we found that OVXHF mice displayed higher biodiversity and a distinct gut microbiome composition compared with normal chow-fed mice. Functional analysis further revealed that genes associated with the iron complex transport system and ATP-binding cassette are differentially expressed in OVXHF mice. Moreover, normal fecal microbiota transplantation (FMT) successfully restored the cognitive impairments in OVXHF mice through rebalancing the gut microbiota. In particular, the *Bacteroides*-to-*Prevotella* ratio was significantly increased in OVXHF mice but rescued by FMT. Altogether, these results suggest that an imbalance between *Bacteroides* and *Prevotella* in the gut microbiota accelerates the process of cognitive dysfunction in postmenopausal women on a high-fat diet.

Keywords: Estrogen deficiency; High-fat diet; Cognition decline; Gut microbiome; *Bacteroides-Prevotella* imbalance

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1. Introduction

Dementia is a pathological neurodegenerative disorder characterized by a progressive decline in cognition. Alzheimer's disease (AD) accounts for 60–80% of all dementia cases, making it the predominant neurodegenerative cause of the condition.[1,2] Increasing evidence suggests that AD affects men and women unequally. It has been reported that after the age of 65, 5.8 million Americans are diagnosed with AD, including 3.6 million women and 2.2 million men.[3] These findings suggest that sex differences matter in the development of AD. One potential explanation for the increased susceptibility of women to AD is menopause, which causes a drastic decline in estrogen levels. Estrogens (including estrone, estradiol, and estriol) are a group of neuroactive steroid hormones related to memory and cognition.[4] As expected, estrogen deprivation of women after menopause can increase the risk of AD, according to a 3-year longitudinal study.[5] Accordingly, studies have found that estrogen hormone replacement therapy can have a protective effect against cognitive decline.[6-8] However, other evidence also shows that

hormone replacement therapy has no benefit in AD, with some cases even indicate a negative effect on cognition.[9,10] Considering the fact that estrogen supplementation fails to consistently relieve AD symptoms and not all aged women develop AD, we wondered whether there were other risk factors underlying the high morbidity of AD in female.

In light of the multifactorial origin of dementia, mounting evidence suggests that lifestyle factors significantly contribute to its progression.[11] Lately, the connection between diet and brain function has become a major focus of research. The Mediterranean diet (MD), which is characterized by high portion of whole grains, veggies, nuts, beans, and olive oil, moderate amount of fish, and limited portion of dairy and meat,[12] is widely considered a major boon to overall health. Extensive research indicates a correlation between adherence to MD and a reduced risk of AD, as well as enhanced cognitive abilities.[13-15] Conversely, a high-fat Western dietary pattern may hasten cognitive impairment.[16,17] Therefore, this raises the question: what distinguishes MD from the Western diet, and how do these differences exert opposite effects on dementia? One of the most noteworthy features of MD is its low-fat content. Therefore, we speculated that a high-fat diet (HFD) may be a contributory factor accelerating cognitive decline in postmenopausal women.

With the rapid development and evolution of new technologies, the microbiota residing in the gastrointestinal tract has been increasingly regarded as the "second brain" that regulates host heath.[18,19] The gut-brain axis, a concept proposed by researchers, describes a bidirectional communication between gut microbiota and the brain, facilitated through the sympathetic and parasympathetic nervous systems, as well as circulating neuro-modulatory molecules and bacterial metabolites.[20,21] Moreover, the microbiome is highly adaptable and susceptible to change due to a range of environmental and host-specific triggers. When it comes to these influencers, dietary habits stand out as the main driver in shaping both the composition and operational aspects of the microbiome.[22] HFD has been associated with an increased abundance in lipopolysaccharide (LPS)-expressing bacteria, resulting in higher LPS levels in both human[23] and mice circulation.[24] Furthermore, lard-rich diet can increase the abundance of *Bacteroides, Turicibacter*, and *Bilophila* spp., promoting white adipose tissue inflammation and insulin insensitivity.[25] However, it remains unknown how HFD in combination with estrogen deficiency affects gut microbiota and cognitive functions.

To explore the role of HFD in facilitating estrogen deficiency-induced cognition impairment, we established an ovariectomized (OVX) mouse model fed with HFD to mimic the situation of postmenopausal women adopting a fat-enriched diet. We then examined the effect of OVX and HFD on the intestinal microbiota. We found that HFD accelerated cognitive decline in OVX mice. Moreover, 16S rDNA sequencing facilitated a comparative analysis of gut microbiota composition and profiles across groups. Finally, we also examined whether normal fecal microbiota transplantation could reverse the cognitive impairment and gut microbiota structure of HFD-fed OVX mice.

2. Materials and methods

2.1. Mice and diet

Juvenile female C57BL/6 mice were obtained from the Animal Center of the Second Affiliated Hospital of Harbin Medical University. Mice were housed in a temperature-controlled room at 25°C under a 12 h light/dark cycle and had ad libitum access to food and water. Under specific pathogen-free conditions, all mice were maintained in a climate chamber with controlled light and humidity (4 mice/chamber), supplied with HEPA-filtered air, and provided with irradiated food and water. The mice were fed either a regular chow diet or an HFD, which consisted of 45% of calories from fat, 35% from carbohydrates, and 20% from protein (HFK Bioscience, #H10045). Body weight was measured weekly. After 8 weeks of feeding, the mice were subjected to behavioral tests or fecal collection. All animal experiments were approved by the Institutional Animal Care and Use Committee of Harbin Medical University and the Institute of Laboratory Animal Science of China. All procedures conformed to the Directive 2010/63/EU of the European Parliament.

2.2. OVX surgery

Two-month-old female mice were anesthetized using an intraperitoneal injection of sodium pentobarbital (100 mg/kg). After prepping the surgical site, we shaved the fur from the flank region, specifically the area nestled between the last rib and just above the pelvis. The skin was then disinfected with chlorhexidine solution before making an incision along the dorsal midline. The muscle layers were carefully separated with curved-tip scissors, and the ovarian fat pad was gently exteriorized through the opening. Hemostatic tweezers were used to firmly clamp the region just below the ovary, and the area to be removed was tied off with two knots using sterile thread, effectively demarcating the surgical field. Following this, the ovary was excised. The same procedure was then replicated on the left side. Following the procedure, the abdominal incision was closed using stainless steel wound clips. The animals were kept under observation in the laboratory until they had fully come around from the anesthesia. After that, they were returned to the animal care facilities and checked on a daily basis.

2.3. Barnes-Maze test

Behavioral assessments using the Barnes maze were conducted following established protocols with minor adjustments.[26] In a nutshell, the mice were subjected to evaluations during the day's light phase, and they were moved into the behavioral testing area an hour ahead of the experiment's kickoff. The Barnes maze featured a clear, elevated, well-lit white circular platform, measuring 91 cm across, and it had 20 evenly spaced, 5 cm wide holes around the edge, with one of those leading to a secret exit. The walls of the testing area were adorned with spatial cues featuring unique patterns and forms. The assessment was broken down into phases of habituation, learning, and the probe test. In the experimental setup, a 500-lux light and an electric fan were employed to generate light, air currents, and noise—key elements to trigger escape responses. On the day of habituation, the research mouse spent 30 seconds acclimatizing to the target box, before being positioned in the center of the apparatus. It was then allowed to roam until it discovered the escape tunnel or the 200-s test session was reached. The acquisition phase entailed a total of 5 days of daily training sessions, with two rounds of trials each day (with a time cap of 3 min per trial; approximately an hour between trials), and the starting point was shuffled among the four quadrants. Each trial began with the mouse placed in a start chamber situated in the middle of one of the quadrants. A 15-s countdown was initiated, and following its conclusion, the start chamber was raised, unleashing the mouse to navigate the maze. The trial comes to a close either when the mouse makes a beeline for the escape tunnel or when the clock strikes 3 min. Should the mouse be unable to locate the escape tunnel within the 3-min window, the researcher will assist it and let it chill in the tunnel for 15 s. Post-trial, the maze and escape tunnel are sanitized with a 75% ethanol solution. Throughout the training phase, the mouse's path, errors, and time taken to locate the target hole were meticulously documented using a video tracking software called SuperMaze (Shanghai XinRuan Software, China). Three days after the final training session, the probe trial takes place, where the escape box is eliminated, and the start chamber is centered in the apparatus. The subject mouse was permitted to locate the target hole within a 3-min window, with the response latency and errors for finding the target hole recorded.

2.4. Novel object recognition test

In accordance with the previously outlined methodology,[27-29] novel object recognition tests (ORTs) were conducted. Before the acclimatization phase, the mice were permitted to roam freely in an open field to become acclimated. Following this, on the day after the habituation period, the mice were given the opportunity to freely investigate two indistinguishable objects—a pair of Lego bricks, 12.5 cm tall, 2.5 cm deep, and 7.5 cm wide, constructed from alternating hues of blue, yellow, red, and green—positioned at predetermined spots within the testing area for a duration of 10 min. To track the duration of exploration for each object, the SuperMaze video-tracking system, developed by XinRuan in Shanghai, China and utilizing nose-point detection technology, was employed. Investigative engagement was defined as olfactory or tactile interaction with an object when the mouse's nose was within 2 cm of the item. At the end of each session, the mice were gently returned to their familiar habitats, and the testing chamber, along with all objects was meticulously sanitized with 75% ethanol solution, followed by a thorough air-drying process for a duration of 3 min. After a 24-h intersession interval (ISI) without intervention, one of the familiar objects was swapped out for a new one—a tissue culture flask that stood at 15.5 cm tall, 3.5 cm deep, and 9 cm across. The following formula was used to determine object preference: Preference percentage = (Time to investigate the particular object/total exploration time for both objects) × 100%. If the overall exploration time was <10 s, the data were not included.

2.5. Fecal microbiota transplantation

Fecal microbiota transplantation was performed as previously described.[30] To deplete native gut microorganisms, OVXHF mice were given 0.2 mL/mouse of triple antibiotics (1.25 mg/L vancomycin, 2.5 mg/L ampicillin, and 2.5 mg/L metronidazole) every day for three consecutive days before transplantation. Then, 200 mg of stool (from Sham mice) was reconstituted in 5 mL of PBS, vortexed for 3 min, and allowed to settle by gravity for 2 min. A 200 μL of the supernatant was then administered to recipient mice by oral gavage to accomplish the transplant. FMT was conducted on OVXHF mice once daily for 30 consecutive days through oral gavage 1 month after OVX surgery and HFD exposure.

2.6. Gut microbiota DNA extraction

Fecal DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen). Approximately 200 mg of mouse fecal sample was mixed with 1 ml of EX Inhibition Buffer and the proper quantity of glass beads (Qiagen) with a 0.5 mm diameter. After that, the sample was homogenized by vortex-mixing twice a minute at 60 Hz. The following procedures were applied to the supernatant after it had been treated with an InhibitEX Tablet. A NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA) was used to measure the amount of DNA.

2.7. 16S rDNA gene sequencing

A universal primer targeting the conserved region of 16S rDNA was constructed as follows: 5'-CCTACGGGRSGCAGCAG-3' (universal primer 341F) and 5'-GGACTACVVGGGTATCTAATC-3' (universal primer 806R) for PCR amplification of the variable region (V3+V4) of the rRNA gene or specific gene fragments. The PCR products were analyzed using the Illumina NovaSeq PE250 system (Illumina, Inc., CA, United States).

2.8. Gut microbiota taxonomical and functional annotation

After sifting through the raw data and removing reads with over 10 substandard bases (those less than Q20) or 15 adapter sequence bases, clean reads were processed in QIIME2 for taxonomic annotation. Operational taxonomic units (OTUs) were clustered at 97% sequence similarity using UPARSE. Functional prediction of microbial communities was performed with PICRUSt2 (v2.3.0) based on ASV data and taxonomic information, yielding KEGG Orthology (KO) pathway distributions for each sample. To measure the alpha diversity across the samples, we used Mothur (v.1.30) and its suite of tools such as the Rarefaction curve, Shannon index, Rank abundance curve, Chao1 richness estimator, and the Simpson diversity index. Beta diversity was analyzed using unweighted UniFrac bandwagon, followed by principal component analysis (PCA), principal coordinates analysis (PCoA), and unweighted pair-group method with arithmetic mean (UPGMA) clustering. Finally, linear discriminant analysis effect size (LEfSe) was applied to assess the impact of the intestinal microbiome.

2.9. Statistical analysis

The results were presented as mean ± standard error of mean. A Student's t-test was applied for statistical comparisons between two groups, whereas a one-way analysis of variance was utilized to assess differences across three or more groups. Tukey's *post hoc* tests were conducted to delve into the significant main effects. Findings were deemed statistically significant when $p<0.05$.

3. Results

3.1. HFD facilitated episodic and spatial memory impairment in OVX mice

To investigate whether HFD could predispose estrogen-deficient mice to cognitive impairment, we subjected female mice to HFD following ovariectomy (OVX) surgery. Previous studies have shown that HFD is always accompanied by overweight and serum lipid disturbances.[31] Interestingly, after 1 month of HFD, we found no differences in body weight or serum lipid levels in any group (Figure 1A-D). Analogous to the above results, there were no differences in latency and error times in the Barnes maze or discrimination index in novel ORT in each group (Figure 1E-R). The above result indicates that there is no episodic and spatial memory impairment after 1 month of HFD in OVX mice.

Even after 2 months of HFD, there was still no differences in body weight and serum lipid levels, including total triglyceride (TG) and total cholesterol (TC), in the Sham+HFD group (Figure 2A-D). However, these parameters were significantly increased in 2-month OVXHF mice, indicating that HFD could affect the body weight and serum lipid levels of estrogen-deficient mice (Figure 2A-D). We also found that 2-month OVXHF mice displayed significantly impaired spatial memory, manifested as extended total distance (Figure 2E-G), prolonged escape latency (Figure 2H and I), and more errors (Figure 2J and K) during probe trial of the Barnes maze test. Notably, no significant difference of average speed was found among the four groups (Figure 2L). As for novel object recognition (NOR), OVXHF mice showed decreased exploration frequency (Figure 2N), time spent with the novel object (Figure 2Q), and a lower discrimination index (Figure 2O-R) on day 2, reflecting attenuated episodic memory. Together, these results showed that HFD could facilitate the episodic and spatial memory impairment in OVX mice.

3.2. HFD induces gut dysbiosis in OVX mice

HFD has been reported to alter the intestinal microbiome and induce cognitive decline through the gut-brain axis.[32,33] To investigate whether HFD induces specific changes in the gut microbial population in OVX mice, we conducted 16S rDNA sequencing to characterize the gut microbiota (GM) under different conditions. After size and quality screening, as well as chimera elimination, we got the sequence length distribution histogram so that optimized sequences for accurate analysis can be obtained (Figure 3A). OTUs were clustered at 97% sequence similarity using Usearch,[34] and 117 OTUs were identified as the core microbiome across all samples (Figure 3B). PCA revealed significant clustering of microbiota across groups (Figure 3C). Although the Sham+HFD and OVX groups showed significant difference in the first and second principal components, respectively (PCA1: 15.91%, PCA2: 9.53%) when compared to Sham group, the two experimental groups were found to be on par with one another, while they markedly differed from OVXHF group. These results indicated that the HFD caused significant changes on GM in OVX mice.

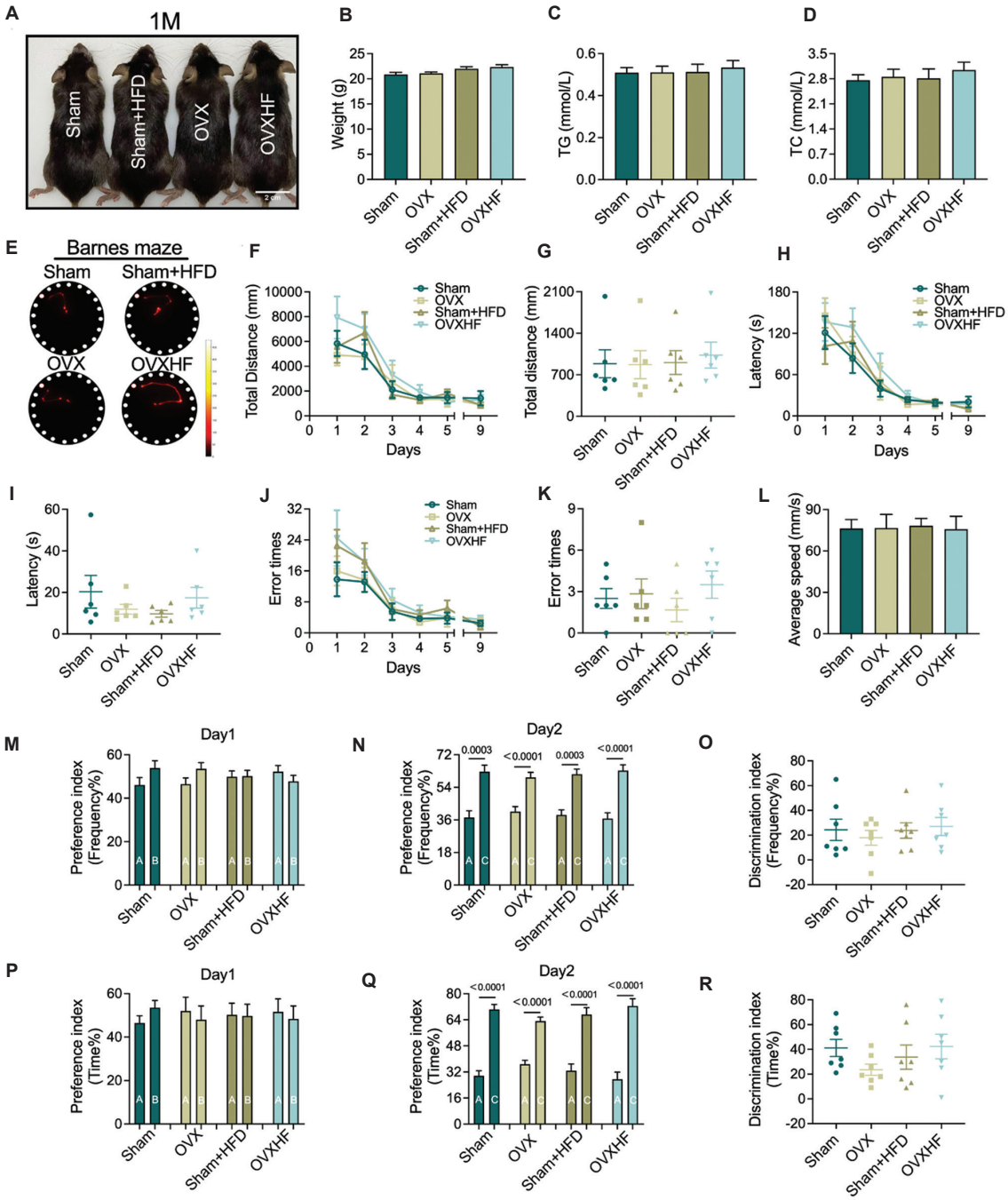


Figure 1. One-month HFD failed to cause cognitive decline in OVX mice. (A) Comparison of the body shape of 1-month (1M) Sham, Sham+HFD, OVX, and OVXHF mice. (B) Body weight at the 4th week for Sham (n = 9), OVX (n = 8), Sham+HFD (n = 8), OVXHF (n = 9). (C) The levels of total glycerol in serum of 1M Sham (n = 6), OVX (n = 6), Sham+HFD (n = 6), OVXHF (n = 6). (D) The levels of total cholesterol in the serum of the above four groups. (E) Schematic diagram of the traces in the Barnes maze test during the probe trial. (F) Total distance during the Barnes maze test for 1M Sham (n = 6), 1M OVX (n = 6), 1M Sham+HFD (n = 6), 1M OVXHF (n = 6). (G) Total distance of the four groups in the Barnes maze during the probe trial. (H) Escape latency during the Barnes maze test of these four groups. (I) Escape latency of the four groups in the Barnes maze during the probe trial. (J) Number of errors in the Barnes maze test. (K) Number of errors in the Barnes maze test during the probe trial. (L) Average moving speed of the four groups during the Barnes maze test. (M) Preference index for frequency on day 1 of 1M Sham (n = 8), OVX (n = 8), Sham+HFD (n = 8), OVXHF (n = 8) in the ORT. (N) Preference index for frequency on day 2 of the above four groups. (O) Discrimination index for the frequency of the four groups. (P) Preference index for contact time on day 1 of the four groups. (Q) Preference index for contact time on day 2 of the four groups. (R) Discrimination index for contact time of the four groups. Data are presented as mean ± SEM and analyzed by one-way ANOVA.

Abbreviations: ANOVA: Analysis of variance; 1 M: 1-month; HFD: High-fat diet; ORT: Object recognition test; OVX: Ovariectomized; OVXHF: HFD-fed OVX; SEM: Standard error of the mean.

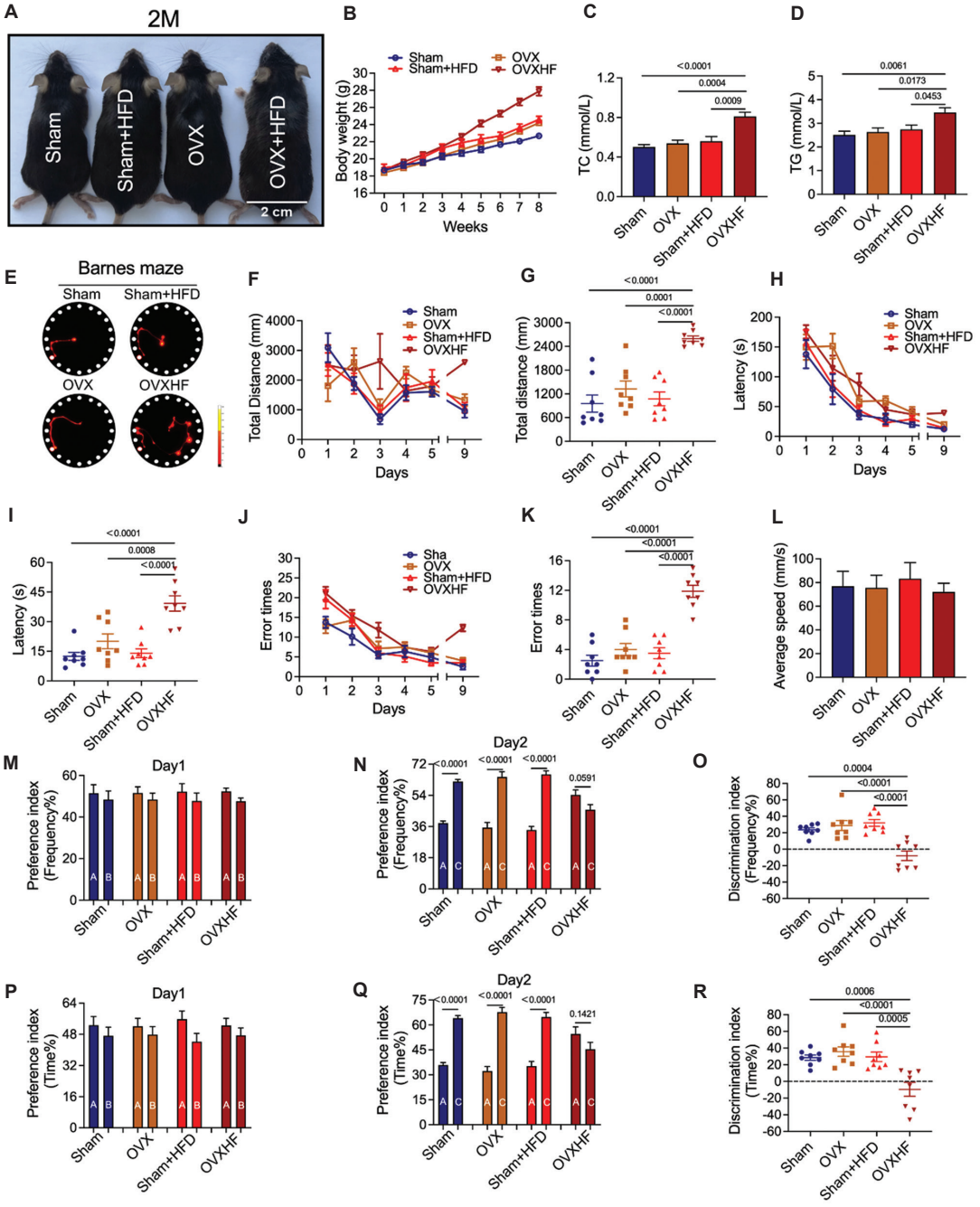


Figure 2. Two-month HFD facilitated cognitive impairment in OVX mice. (A) Comparison of the body shape of 2-month (2M) Sham, Sham+HFD, OVX, and OVXHF mice. (B) Weekly body weight of Sham (n = 9), OVX (n = 8), Sham+HFD (n = 8), OVXHF (n = 9) mice. (C) The levels of total cholesterol in the serum of 2M Sham, OVX, Sham+HFD, and OVXHF. n = 6 for each group. (D) The levels of total glycerol in the serum of the above four groups. (E) Schematic diagram of the traces in the Barnes maze test during the probe trial. (F) Total distance during the Barnes maze test for each group (n = 8). (G) Total distance of the four groups in the Barnes maze during the probe trial. (H) Escape latency during the Barnes maze test of the four groups. (I) Escape latency during the Barnes maze test of the four groups. (J) Number of errors in the Barnes maze test. (K) Number of errors in the Barnes maze test during the probe trial. (L) Average moving speed of the four groups during the Barnes maze test. (M) Preference index for frequency on day 1 of 2M Sham (n = 8), OVX (n = 8), Sham+HFD (n = 8), OVXHF (n = 8) mice in the ORT. (N) Preference index for frequency on day 2 of the above four groups. (O) Discrimination index for the frequency of the four groups. (P) Preference index for contact time on day 1 of the four groups. (Q) Preference index for contact time on day 2 of the four groups. (R) Discrimination index for contact time of the four groups. Data are presented as mean ± SEM and analyzed by one-way ANOVA.

Abbreviations: ANOVA: Analysis of variance; HFD: High-fat diet; ORT: Object recognition test; OVX: Ovariectomized; OVXHF: HFD-fed OVX; SEM: Standard error of the mean; 2M: 2 months.

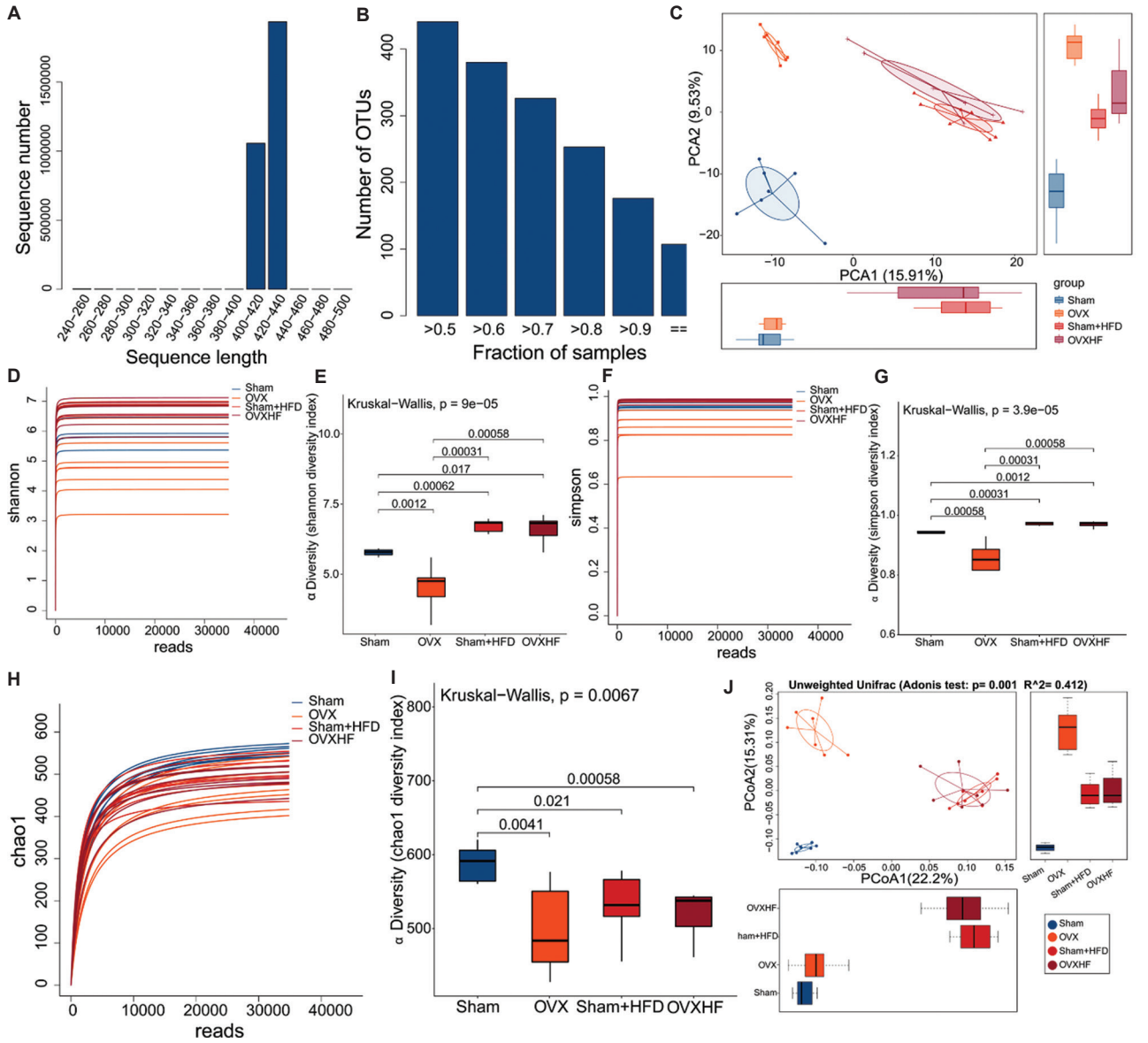


Figure 3. HFD altered the alpha and beta diversities of gut microbiota in OVX mice. (A) The sequence length distribution histogram of 16S rDNA sequencing. (B) Core microbiome that covers 100% samples. (C) Principal component analysis (PCA) of the microbiota composition in 2-month Sham ($n = 7$), OVX ($n = 7$), Sham+HFD ($n = 8$), and OVXHF ($n = 7$) mice. (D–G) Shannon and Simpson indices of 16S rDNA sequencing indicated community diversity in the four groups. (H–I) Chao1 index of 16S rDNA sequencing shows community richness in the above four groups. (J) Principal coordinates analysis (PCoA) based on unweighted UniFrac analysis of the Adonis test shows beta-diversity of these four groups. Each symbol represents a single fecal sample. Data are presented as mean ± SEM and analyzed by one-way ANOVA.

Abbreviations: HFD: High-fat diet; OVX: Ovariectomized; OVXHF: HFD-fed OVX; SEM: Standard error of the mean; ANOVA: Analysis of variance.

Alpha diversity reflects both sample diversity, represented by indices like Shannon and Simpson, and species richness, such as Chao1.[35] Compared to the Sham group, Shannon and Simpson indices were significantly reduced in the OVX-only group. However, in the OVXHF mice, these indices bounced back, mirroring the levels seen in the Sham+HFD group. This suggests that HFD reshapes gut bacterial diversity in OVX mice, counteracting the diversity loss caused by estrogen deficiency (Figure 3D-G). The Chao1 indices were significantly decreased in the Sham+HFD, OVX, and OVXHF groups compared to the Sham group, but there were no significant differences among them. This indicates that both HFD and OVX can decrease gut bacterial richness; however, HFD does not affect the gut bacterial richness in OVX mice (Figure 3H and I). PCoA of Aitchison distances further

revealed significantly altered GM composition in OVX, Sham+HFD, and OVXHF groups compared to the Sham control, while the Sham+HFD and OVXHF groups were of similar composition (Figure 3J). Accordingly, these results suggest that HFD can change the GM composition and induce GM dysbiosis in OVX mice.

3.3. The ratio of *Bacteroides/Prevotella* increased in OVXHF mice

To further clarify the effects of HFD and estrogen depletion on intestinal flora at the genus level, bacterial makeup was examined thoroughly. LEfSe analysis was used to present the bacteria that played an important role in Sham, OVX, Sham+HFD, and OVXHF mice (Figure 4A). Bar plots presented a comprehensive breakdown of the prevalence of various genera across the 29 fecal samples, with *Alloprevotella, Prevotella, Lactobacillus, Clostridium XIVaI,* and *Bacteroides* dominating in the four groups (Figure 4B). Then, we analyzed the explicit alterations of these bacterial genus, respectively. In total, we identified 6 genera significantly changed in Sham+HFD, OVX, or OVXHF mice, respectively (Figure 4C-H). Notably, *Alloprevotella* abundance was increased in OVX mice compared to the Sham group but significantly reduced in both Sham+HFD and OVXHF mice (Figure 4C). *Streptococcus* was the only genus consistently decreased in all three groups (Figure 4D). The level of *Turicibacter* and *Bifidobacterium* was both elevated in OVXHF mice but not OVX and Sham+HFD mice (Figure 4E and F). The relative prevalence of *Prevotella* in OVX and OVXHF mice mirrored that of *Alloprevotella*, while stayed unchanged in Sham+HFD mice (Figure 4G). The level of *Bacteroides* was significantly upregulated in Sham+HFD and OVXHF mice (Figure 4H). This study indicates that the human gut microbiota can be categorized into three distinct enterotypes, primarily characterized by *Bacteroides*, *Prevotella*, or *Clostridiales* organisms.[36] Thus, we wondered whether the ratio of *Bacteroides/Prevotella* (B/P) was associated with the cognitive impairment caused by HFD and OVX co-exposure. OVX alone failed to cause disturbance on the B/P ratio while HFD slightly increased this ratio (Figure 4I). Interestingly, the combination of HFD with OVX led to dramatic elevation of the B/P ratio (Figure 4I). In conclusion, HFD reshaped the microbial spectrum in OVX mice, and the rise of B/P ratio may represent a key microbial signature associated with cognitive impairment under combined HFD and estrogen deficiency.

3.4. Microbial functional alterations in the OVXHF mice

After dissecting the bacterial composition across groups, we further explored the enrichment of KO functions in OVXHF mice. By comparing the 16S rDNA gene sequences against genomic reference databases, the functional potential of the microbial community was predicted. LEfSe analysis was employed to assess the impact of KO genes from each group on the differential outcomes and to identify corresponding KO genes responsible for notable differential effects (Figure 5A). Among these, only the ATP-binding cassette (ABC)-2 type transport system permease protein was increased in OVX, Sham+HFD, and OVXHF group versus Sham group (Figure 5B). Subfamily B of the ABC proteins showed a modest rise in the OVX cohort versus the Sham, whereas HFD induced a notable drop in the ABC subfamily B levels in OVX mice (Figure 5C). Furthermore, genes belong to the iron complex transport system, RNA polymerase sigma-70 factor, and 3-oxoacyl- [acyl-carrier protein] reductase were remarkably decreased in OVX group in contrast to Sham group (Figure 5D-G, K). Comparatively, expression of these genes demonstrates a reverse pattern in the OVXHF cohort relative to OVX mice (Figure 5D-G, K). In addition, the abundance of cold shock protein, methyl-accepting chemotaxis protein, and 2,3-biphosphoglycerate-dependent phosphoglycerate showed no obvious difference between Sham and OVX group, but HFD separately increased or decreased the abundance of these genes in the OVX group (Figure 5H-J). Taken together, the results suggest that HFD causes disturbance in the functional structure of GM in OVX mice which may account for the cognitive impairment induced by HFD.

In addition, we investigated the association between bacterial genera and host physiological or behavioral parameters. LEfSe correlation analysis revealed that the weight of mice was positively correlated with *Elusimicrobium*, *Blautia*, and *Christensenella* but negatively correlated with *Odoribacter*, *Mycoplasma*, *Clostridium XVIII*, *Sporobacter*, and *Paraprevotella*. Barnes maze errors showed positive associations with *Desulfovibrio* and *Turicibacter* but inversely correlated with *Prevotella*, *Alloprevotella*, and *Streptococcus*. We also identified the genera that are closely correlated with the levels of 5-hydroxytryptamine (5-HT), L-Kynurenine (L-KYN) and L-Tryptophan (L-TRP), which are indispensable nutrients for the hosts (Figure 5L).

3.5. Fecal microbiota transplantation restored cognitive impairment in OVXHF mice

Our findings demonstrate that HFD accelerates memory decline in OVX mice while disrupting both the composition and functional capacity of the gut microbiota. However, it is still not clear whether these gut imbalances are the culprit behind the cognitive decline. To address this, we introduced healthy murine gut microbiota into

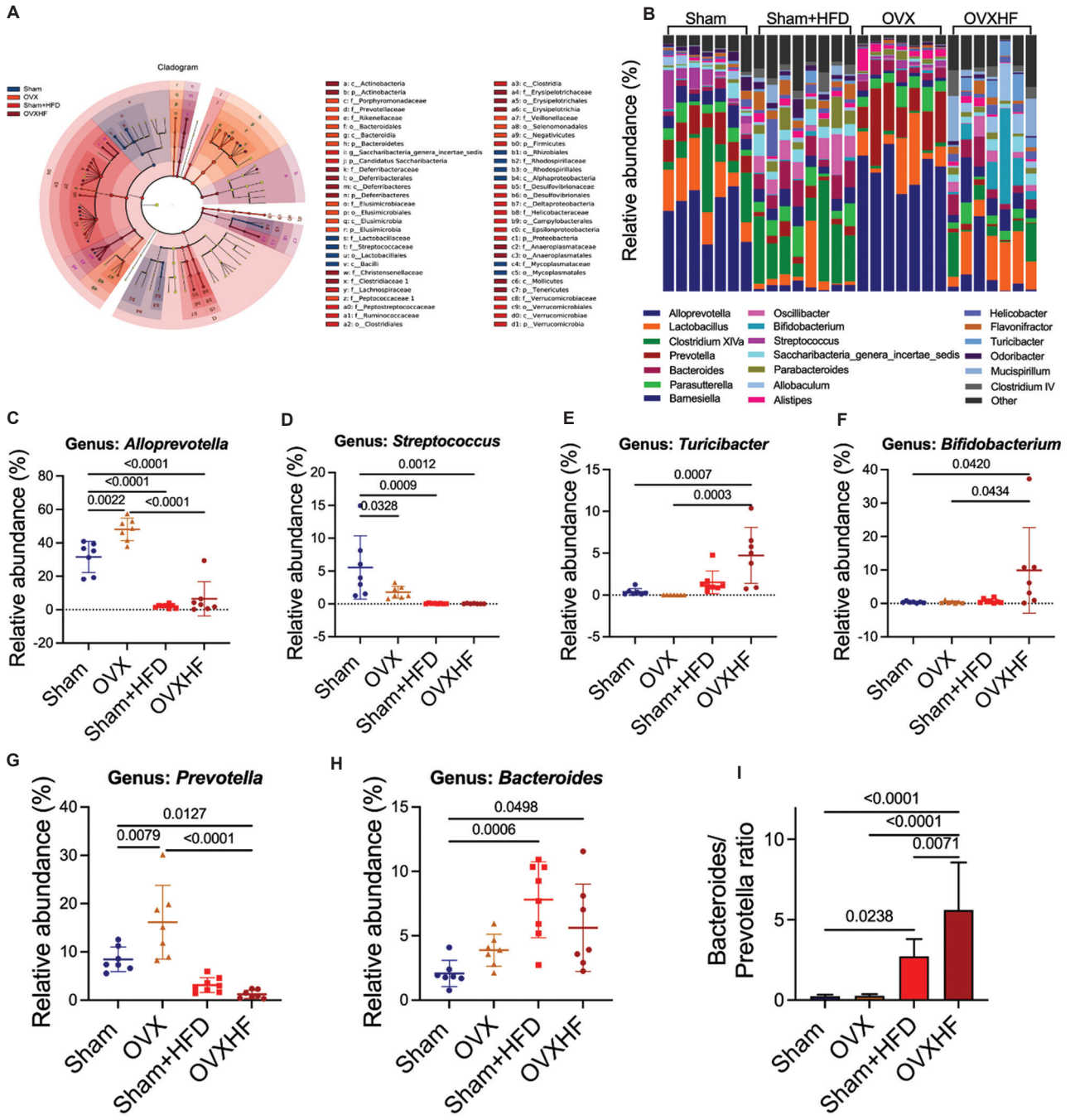


Figure 4. Characterization of gut microbiota composition at the genus level. (A) Linear discriminant analysis (LDA) effect size (LEfSe) analysis showing the bacteria that played an important role in 2-month Sham (n = 7), OVX (n = 7), Sham+HFD (n = 8), and OVXHF (n = 7) mice. (B) Proportions of different bacterial genus in the gut microbiota of the above four groups. Each column represents one mouse sample. (C–H) The bacterial genus that were dysregulated by OVX and HFD, including *Alloprevotella*, *Prevotella*, *Streptococcus*, *Bacteroides*, *Turicibacter,* and *Bifidobacterium*. (I) The ratio of *Bacteroides* and *Prevotella* in the four groups. Data are presented as mean ± SEM and analyzed by one-way ANOVA.
Abbreviations: ANOVA: Analysis of variance; HFD: High-fat diet; OVX: Ovariectomized; OVXHF: HFD-fed OVX; SEM: Standard error of the mean.

the digestive systems of OVXHF mice through oral administration. FMT alleviated weight gain and serum TC and TG levels in OVXHF mice (Figure 6A-D). Moreover, the Barnes maze task showed that OVXHF mice receiving FMT exhibited markedly improved path length, latency, and error times in locating the escape aperture (Figure 6E-L). In ORT study, FMT notably augmented OVXHF mice interaction duration and rate with the novel

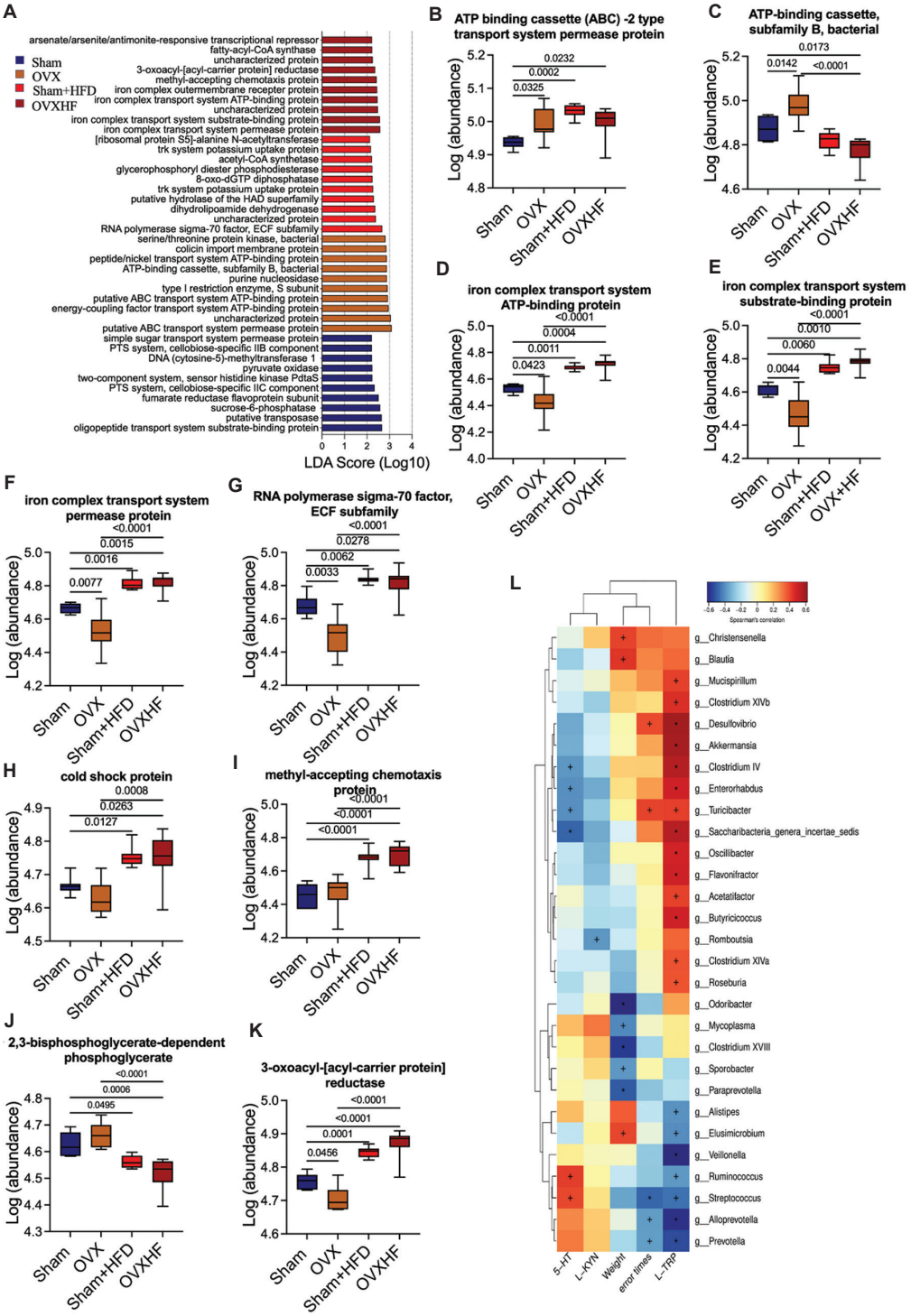


Figure 5. KEGG ortholog functional analysis of gut microbiota in Sham, OVX, Sham+HFD, and OVXHF mice. (A) LEfSe analysis was used to find the counterpart KEGG ortholog (KO) genes that produce significant differential effects (default screening criteria LDA >2). The ordinate represents the log value obtained by LDA for KO genes with a significant effect in the 2-month Sham, OVX, Sham+HFD, and OVXHF groups. (B–K) The KO genes that were dramatically altered by OVX and HFD in Sham ($n = 7$), OVX ($n = 7$), Sham+HFD ($n = 8$), and OVXHF ($n = 7$) mice. (L) LEfSe analysis revealed the correlation of various genera with body weight, error times, 5-HT, L-KYN, and L-TRP levels. Data are presented as mean ± SEM and analyzed by one-way ANOVA. $^{+}p<0.05$; $^{*}p<0.01$.

Abbreviations: ANOVA: Analysis of variance; HFD: High-fat diet; LDA: Linear discriminant analysis; LEfSe: LDA effect size; OVX: Ovariectomized; OVXHF: HFD-fed OVX; SEM: Standard error of the mean.

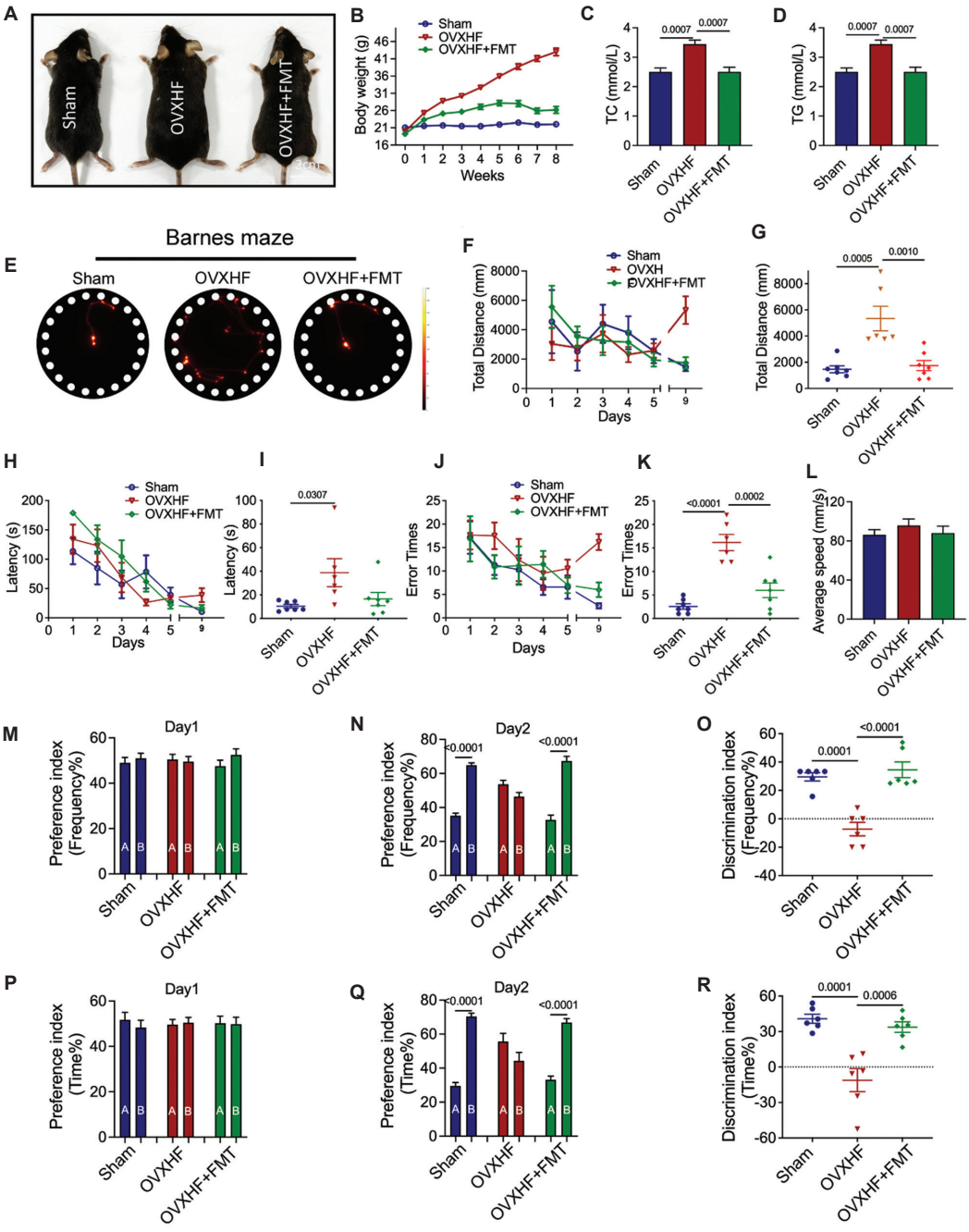


Figure 6. Fecal microbiota transplantation restored the spatial and episodic memory impairment of OVXHF mice. (A) Comparison of the body shape of 2M Sham, OVXHF, and OVXHF+FMT mice. (B) Weekly body weight of Sham (n = 7), OVXHF (n = 6), and OVXHF+FMT (n = 7) mice. (C) The levels of total cholesterol in the serum of 2-month Sham, OVXHF, and OVXHF+FMT mice. n = 6 for each group. (D) The levels of total glycerol in the serum of the above four groups. (E) Schematic diagram of the traces in the Barnes maze test during the probe trial. (F) Total distance during the Barnes maze test for Sham (n = 7), OVXHF (n = 6), and OVXHF+FMT (n = 7) mice. (G) Total distance of the four groups in the Barnes maze during the probe trial. (H) Escape latency during the Barnes maze test of these four groups. (I) Escape latency during the Barnes maze test of these four groups. (J) Number of errors in the Barnes maze test. (K) Number of errors in the Barnes maze test during the probe trial. (L) Average moving speed of the four groups during the Barnes maze test. (M) Preference index for frequency on day 1 of 2M Sham, OVXHF, and OVXHF+FMT mice. n = 6 for each group. (N) Preference index for frequency on day 2 of the above four groups. (O) Discrimination index for frequency of the four groups. (P) Preference index for contact time on day 1 of the four groups. (Q) Preference index for contact time on day 2 of the four groups. (R) Discrimination index for contact time of the four groups. Data are presented as mean ± SEM and analyzed by one-way ANOVA.

Abbreviations: ANOVA: Analysis of variance; FMT: Fecal microbiota transplantation; OVXHF: High-fat diet-fed ovariectomized mice; SEM: Standard error of the mean; 2M: 2 months.

item, along with elevating the novelty preference score (Figure 6M-R). Together, these results indicate that FMT can reverse the obesity phenotype and spatial and episodic memory impairments in OVXHF mice. Concurrently, our study supports the notion that HFD exacerbates cognitive decline in OVX mice through intestinal dysbiosis.

3.6. Fecal microbiota transplantation restored intestinal bacterial structure in OVXHF mice

To further investigate whether the beneficial effects of FMT on cognitive function in OVXHF mice were linked to the altered functional structure of their gut microbiota, we re-employed 16S rDNA sequencing to pinpoint the specific shifts in gut microbiota following FMT. First, goods coverage indicated that the sequencing exhibits excellent coverage (Figure 7A). The Shannon and Simpson indices were increased in OVXHF and FMT-treated OVXHF mice than in Sham mice, but no significant difference was observed between the two groups (Figure 7B-C). However, the Chao1 index was reduced in OVXHF mice, whereas FMT application dramatically increased the Chao1 index in OVXHF mice (Figure 7D). These results indicated that FMT significantly restored the richness of OVXHF mice. Analysis on the genus level constructure showed that microbial composition in OVXHF mice was altered after FMT administration (Figure 7E). Then, we further analyzed the abundance of these bacterial genus and identified 7 genera that changed by FMT. In particular, FMT restored the abundance of *Prevotella* and *Parabacteroides* in OVXHF, while increasing the abundance of *Bacteroides, Oscillibacter, Alistipes*, and *Flavonifractor* (Figure 7F). In accordance with the restoration of cognitive impairment by FMT, the B/P ratio was significantly decreased in the OVXHF mice supplied with normal gut flora (Figure 7G), further supporting its role in the cognition decline caused by HFD and estrogen deficiency.

Similarly, we also performed the KO functional analysis in FMT-treated OVXHF mice. The results indicated that the level of ABC, putative ABC transport system permease protein, and 2,3-biphosphoglycerate mutase was restored by FMT administration (Figure 8A-C). In addition, genes related to ABC transport system, peptide/nickel transport system, and LacI family transcriptional regulator were markedly enriched in FMT-treated OVXHF mice (Figure 8D-J). Taken together, these functional genes might be associated with the amelioration of cognitive impairment caused by OVXHF.

4. Discussion

Clinical evidence shows that not all women experience rapid cognitive decline during menopause, though the underlying reasons remain unclear. Dietary patterns affect the cognitive process,[37] and it is speculated that different diet may participate in the process of rapid cognitive decline in menopausal women. In this study, we found that HFD could accelerate cognitive impairment in 2-month OVX mice rather than 1-month OVX mice. Moreover, 16S rDNA sequencing revealed considerable shifts in both the makeup and operation of the gut microbiota in 2-month OVXHF mice. Importantly, FMT effectively restored microbial balance and reversed cognitive decline in these OVXHF mice. Particularly, an increase in *Bacteroides/Prevotella* (B/P) ratio may account for the cognitive impairment in OVXHF mice. The findings clearly indicate that the HFD is a critical risk factor for AD in post-menopausal women, suggesting that early dietary interventions could be a potential strategy to prevent cognitive deterioration in perimenopausal women.

The greater incidence of AD among females compared to males has sparked significant concern.[38] Previous studies have focused on the fluctuation of estrogen levels after menopause and they actually identified a close relation between AD and estrogen deficiency.[39] However, not all women experience cognition decline after menopause, and the disease initiation varies among individuals. Moreover, our previous study found that female mice developed episodic memory impairment after 3 months of estrogen deficiency and spatial memory decline after 5 months of estrogen deficiency,[40] suggesting that additional risk factors may contribute to cognitive deterioration in postmenopausal females. HFD, an unhealthy dietary habit, has been found to exert a more severe effect in female AD mice rather than male AD mice.[41] Thus, we hypothesized that HFD might be a potential risk factor accelerating cognitive impairment in postmenopausal females. However, in this study, 1-month HFD failed to induce cognitive impairment in OVX mice. Instead, after 2-month HFD, OVX mice showed impaired spatial and episodic memory, indicating that 2-month HFD could expedite the cognitive decline in OVX mice. Considering these findings, maintaining a healthy diet may help mitigate cognitive decline after menopause.

Although estrogen levels decline sharply during menopause in women compared to the gradual reduction of testosterone in age-matched men,[42] the abrupt loss of estrogen after OVX still cannot perfectly simulate natural menopause process concomitant with exaggerate physiological and behavioral effects. In addition, other ovarian hormones such as testosterone may also be depleted after OVX, which serves as another defect of this model.[43] Thus, our future plan will continue to study the mechanism of cognitive impairment in perimenopausal women using the natural aging mouse model.

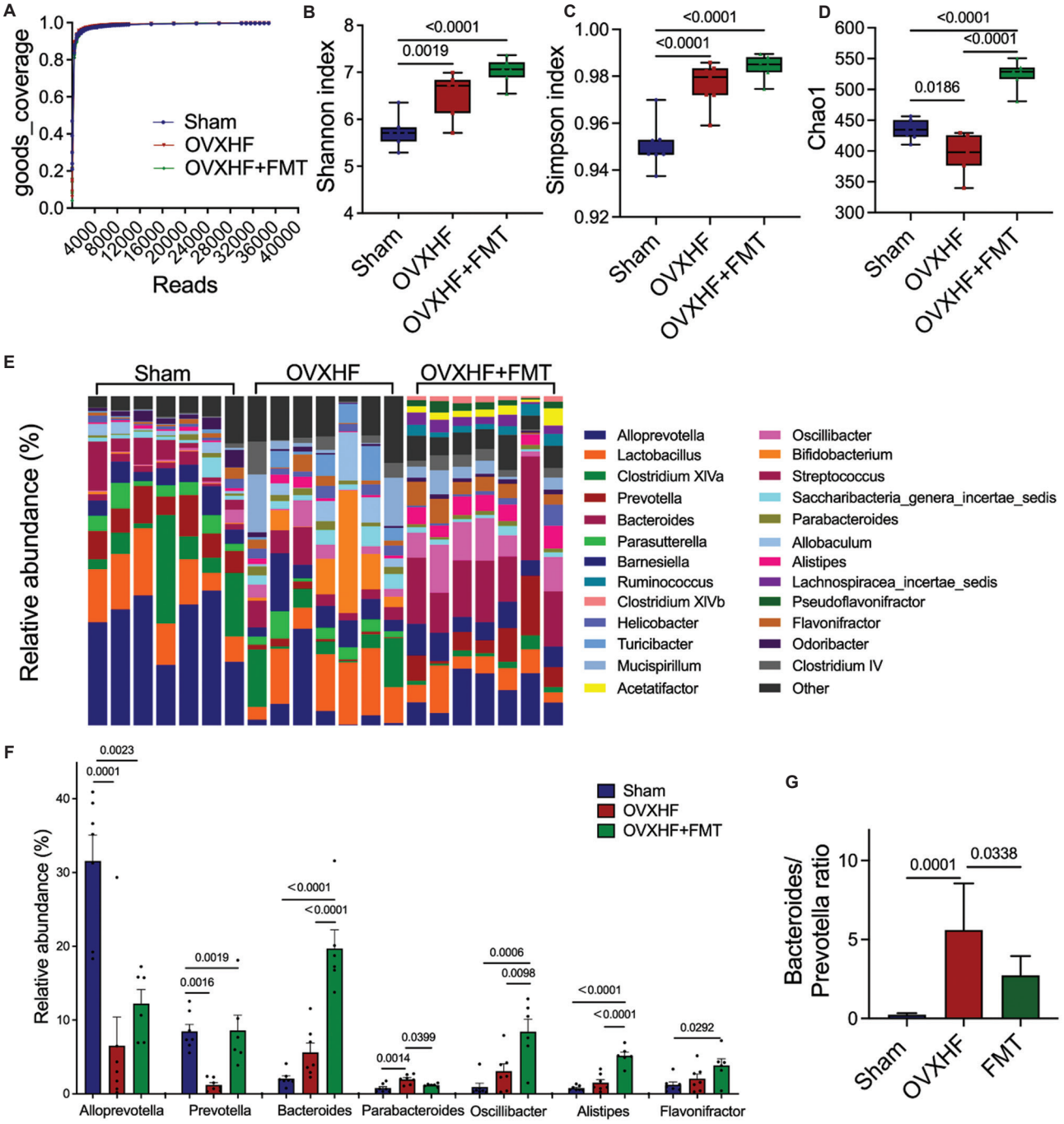


Figure 7. Gut bacteria alterations after fecal microbiota transplantation in OVXHF mice. (A) The goods coverage index of 16S rDNA sequencing. (B–D) Alpha diversity represented by Shannon, Simpson, and Chao1 indices of 16S rDNA sequencing for 2-month Sham (n = 7), OVXHF (n = 7), and OVXHF+FMT (n = 7) mice. (E) Proportional abundance of gut microbiota from the above three groups at the genus level. Each column indicates one mouse sample. (F) The bacterial genus which relative abundance was rectified after FMT, including *Alloprevotella*, *Prevotella*, *Bacteroides*, *Parabacteroides*, *Oscillibacter*, *Alistipes*, and *Flavonifractor*. (G) The *Bacteroides*/*Prevotella* ratio in the Sham (n = 7), OVXHF (n = 7), and OVXHF+FMT (n = 6) mice. Data are presented as mean ± SEM and analyzed by one-way ANOVA.

Abbreviations: ANOVA: Analysis of variance; FMT: Fecal microbiota transplantation; OVXHF: High-fat diet-fed ovariectomized mice; SEM: Standard error of the mean.

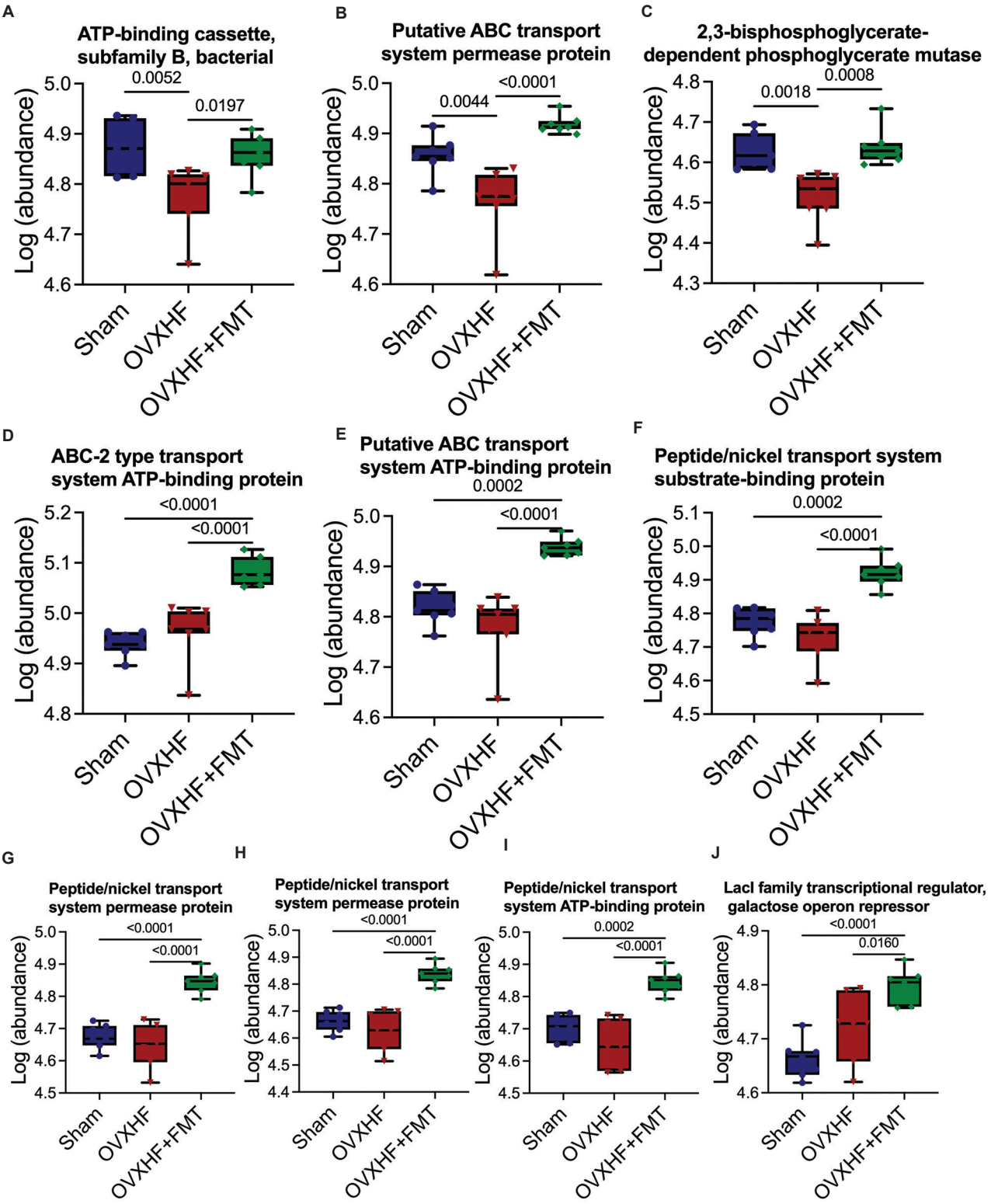


Figure 8. KEGG ortholog functional analysis of gut microbiota in Sham, OVXHF, and OVXHF+FMT mice. (A–C) The abundance of ATP-binding cassette, subfamily B, putative ABC transport system permease protein, and 2,3-biphosphoglycerate-dependent phosphoglycerate mutase was restored after FMT in OVXHF mice. n = 7 for each group. (D–J) FMT significantly increased the abundance of the ABC transport system family, peptide/nickel transport system family, and LacI family transcriptional regulator, galactose operon repressor. Data are presented as mean ± SEM and analyzed by one-way ANOVA Abbreviations: ANOVA: Analysis of variance; FMT: Fecal microbiota transplantation; OVXHF: High-fat diet-fed ovariectomized mice; SEM: Standard error of the mean.

In recent years, GM and its role in neurodegenerative diseases have attracted considerable attention.[44] HFD can effectively affect GM composition and increase the risk of AD.[45] However, whether and how HFD could bring about gut dysbiosis in OVX mice remains unclear. In this study, we profiled the gut microbiome of OVXHF mice through 16S rDNA sequencing techniques and identified 6 genera that exhibited notable shifts among which the abundance of *Prevotella* was dramatically increased in OVX mice while decreased in Sham+HFD and OVXHF mice. Studies have discovered an inverse relationship between *Prevotella* and cognitive impairment.[46] *Bacteroides* is a probiotic that provides the host with volatile fatty acids serving as an energy source.[47] This study revealed a heightened prevalence of *Bacteroides* in Sham+HFD and OVXHF groups, a finding that appears inconsistent with some earlier findings. However, *Prevotella*- and *Bacteroides*-dominated gut flora have been identified as two major enterotypes of human which are closely associated with diet patterns.[36] Thereinto, the *Bacteroides*-dominated enterotype mainly appears in people with high-fat/high-sugar "Western" diet, whereas the *Prevotella*-dominant enterotype is found in people possessing a fiber-rich plant-based diet pattern.[48,49] In this study, the B/P proportion within the gut microbiota of mice was analyzed, revealing a moderate rise in the HFD group and a marked increase in the OVXHF group. Furthermore, normal gut microbiota transplantation successfully restored the B/P ratio. Our findings align with previous studies and suggest that a balance between *Bacteroides* and *Prevotella* is essential for maintaining host neuropsychiatric health.

Beyond genus-level changes, differential genera were correlated with environmental factors. We found that *Prevotella, Alloprevotella*, and *Streptococcus* were negatively correlated with error times in the Barnes maze, implying these genera might account for the spatial memory impairment of OVXHF mice. Tryptophan, or TRP, is an essential amino acid, in which imbalances in TRP's metabolic process play a significant role in the advancement of certain neurological disorders, including depression, AD, and glioma.[50] Here, we examined links between intestinal bacteria and L-TRP, along with its downstream products 5-HT and L-KYN. *Prevotella, Alloprevotella*, and *Streptococcus* were negatively correlated with L-TRP, while *Streptococcus* alone was positively correlated with 5-HT. These results suggest that these three genera may participate in the pathological process of cognitive deficit through disruption of TRP metabolism.

Closer examination of the results revealed that the variation trends of GM were surprisingly consistent in the Sham+HFD and OVXHF mice but different from that in OVX mice, suggesting that HFD exerts a stronger influence on GM than estrogen deficiency. However, even though the Sham+HFD and OVXHF mice shared similar characteristics of GM, Sham+HFD mice failed to develop cognitive impairment. This might be attributed to the complexity of the GM composition and the absence of estrogen deficiency which could also cause lipid and glucose metabolic disorders.[51] Thus, HFD alone was insufficient to induce cognitive decline, whereas co-exposure with estrogen deficiency led to pronounced spatial and episodic memory impairments. The interaction between HFD and estrogen deficiency on host physiology is complicated and interesting, warranting further investigation.

5. Conclusion

Taken together, HFD serves as a critical risk factor that facilitates cognitive decline in post-menopausal women through gut dysbiosis. Normal fecal microbiota transplantation effectively restored the cognitive function caused by HFD and estrogen deficiency, with the imbalance between *Bacteroides* and *Prevotella* likely playing a pivotal role. Thus, further studies should focus on elucidating the underlying mechanisms linking this imbalance in cognitive function and exploring the feasibility of GM-based therapeutic approaches for AD.

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Conflict of interest

Jing Ai is an Editorial Board Member of this journal but was not in any way involved in the editorial and peer-review process conducted for this paper, directly or indirectly. Separately, other authors declared that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Author contributions

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Investigation: Lu Zeng, Xiaobin An, Jing Ma, Guitian Cong, Meijie Chen, Yang Qu, Yan Wu, Xuqiao Wang, Jinan Yang
Methodology: Lu Zeng, Xiaobin An, Wentao Xu, Jing Ma
Writing–original draft: Lu Zeng, Wentao Xu
Writing–review & editing: Jing Ai, Lu Zeng, Yang Qu, Xiaobin An

Ethics approval and consent to participate

All animal procedures were approved by the Institutional Animal Care and Use Committee at Harbin Medical University (no. HMUIRB-2008-06) and the Institute of Laboratory Animal Science of China (A5655-01). All procedures conformed to Directive 2010/63/EU of the European Parliament.

Consent for publication

Not applicable.

Availability of data

Data are available from the corresponding author upon reasonable request.

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ORIGINAL RESEARCH ARTICLE

Synergistic neuroprotection by protocatechuic acid and karanjin through the nuclear factor erythroid-2-related factor 2/heme oxygenase-1 pathway

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Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disorder, and the relationship between oxidative stress and PD is complex and multifaceted. Recent research has demonstrated that protocatechuic acid (PCA) and karanjin (KJN) can mitigate oxidative stress and may offer neuroprotective benefits in PD. PCA has also been reported to work synergistically with various other drugs or phytoconstituents, such as PCA and ginkgolide B in neuroprotection, PCA with catechin and vanillic acid against bacterial adhesion, and PCA with 5-fluorouracil in cancer therapy. 1-methyl-4-phenylpyridinium (MPP^+) was used to induce PD in SH-SY5Y cells, leading to increased intracellular reactive oxygen species (ROS) generation and significant cell damage. Treatments with KJN and PCA reduced MPP^+-induced cell damage by decreasing ROS generation. KJN and PCA enhanced *NRF2* mRNA and heme oxygenase-1 (HO-1) expression. However, the HO-1 inhibitor zinc protoporphyrin diminished the antioxidant and neuroprotective benefits of KJN and PCA. *NRF2* knockdown decreased HO-1 expression and the neuroprotective effects of KJN and PCA. The phosphoinositide 3-kinase/Akt inhibitor LY294002 eliminated the impact of KJN and PCA on *NRF2*-HO-1 expression, cell survival, lactate dehydrogenase release, and ROS production in MPP^+-stimulated SH-SY5Y cells, but not the MAPK pathway inhibitors (PD98059, SP600125, and SB203580). These findings suggest that the neuroprotective effects of KJN and PCA in SH-SY5Y cells are linked to *NRF2*-mediated HO-1 expression, specifically through the PI3K/Akt pathway.

Keywords: Karanjin; Protocatechuic acid; 1-Methyl-4-phenylpyridinium; Parkinson's disease; Neuroprotective effects; Phosphoinositide 3-kinase/protein kinase B pathway

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1. Introduction

Parkinson's disease (PD) causes motor dysfunction, cognitive challenges, speech problems, and neurodegeneration in the human body. The current therapy for PD

focuses on slowing the clinical progression, controlling symptoms, maintaining daily functioning, and preventing motor complications.[1] Dopamine agonists are the only prescribed drugs for PD.[2] Only a few medicines have been effective against neurodegeneration. Recent efforts have been undertaken to identify novel compounds that can protect dopaminergic (DA) neurons from damage caused by various pathogenic processes. While the exact mechanisms behind the selective degeneration of DA neurons remain unclear, increasing evidence indicates that oxidative stress plays a significant role in the death of these neurons.[3] Oxidative stress occurs when there is an imbalance between the generation of reactive oxygen species (ROS) and the function of cellular antioxidants, acting as a common pathogenic mechanism in various diseases. In patients with PD and in animal models, increased ROS levels have been found in both the serum and the substantia nigra.[4] Increased ROS production is thought to be linked to mitochondrial insufficiency, DA metabolism, and neuroinflammation in PD.[5] Reactive species inactivate biological macromolecules, disrupt organelle function, and cause cell death, resulting in the loss of DA neurons in the substantia nigra.[6,7] Studies have demonstrated that inhibiting the formation of ROS helps reduce neuronal damage and PD symptoms.[8] Antioxidant stress is now being considered as one of the primary approaches to treat PD-associated complications.[9] Another antioxidative strategy is to boost cellular antioxidant activity in addition to reducing ROS generation. Heme oxygenase 1 (HO-1) is an antioxidant enzyme that has recently been identified as a crucial antioxidant defense against ROS.[10] The HO isoform, HO-1, catalyzes the conversion of heme to carbon monoxide, iron, and bilirubin, all of which are antioxidants. HO-1 has been linked to a variety of diseases, including neurodegenerative illnesses.[11] In recent *in vivo* and *in vitro* investigations, upregulation of HO-1 expression exhibited neuroprotective benefits and relieved PD symptoms.[12,13] Furthermore, interventions that stimulate the signaling pathways regulating *HO-1* gene expression reduce DA neuron damage and improve PD symptoms.[14] The findings indicate that HO-1 plays an essential role in the treatment of PD. For example, SH-SY5Y and PC12 cells express DA neuron-associated genes and are widely employed to create PD cell models.[15,16] Toxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA), are used to induce neuronal injury in PD models.[16,17] In the body, MPTP is converted to 1-methyl-4-phenylpyridinium (MPP^+), which produces high amounts of ROS that cause oxidative damage to DA neurons. MPP^+ is used to produce PD cell models *in vitro*.[18]

Phytoconstituents have long been used in medicine.[19] A variety of natural substances are utilized to treat neurodegenerative disorders, including PD, because of their antioxidative and anti-inflammatory properties.[20-24] Protocatechuic acid (PCA) is a distinct molecule with a wide array of biological activities. It is found in many plants and foods, which likely contributes to some of their health benefits. It is reported to have various biological properties, such as antioxidant,[21] anti-inflammatory,[22] anticancer,[23] neuroprotective,[24] antidiabetic,[25] and cardioprotective[26] properties. PCA has also been reported to exert synergistic action with various other drugs or phytoconstituents; for example, PCA and ginkgolide B for neuroprotection,[27] PCA with catechin and vanillic acid against bacterial adhesion,[28] and PCA and 5-fluorouracil in cancer therapy.[29] Karanjin (KJN) belongs to the class of furanoflavonoids and is characterized by a unique tricyclic structure. It is primarily found in the seeds and oil of *Pongamia pinnata*. It is reported to have various activities, such as antioxidant[30] and anti-inflammatory,[31] antimicrobial,[32] antidiabetic,[33] gastroprotective,[34] anticancer,[35] antiulcer,[36] and anti-psoriatic activity.[37] The present study was also designed to test the hypothesis that PCA may act synergistically with KJN to produce neuroprotection in PD. In this research, PCA and KJN are proposed as potential agents to inhibit the activation of NF-κB and prevent the death of DA neurons triggered by 6-OHDA in the rat substantia nigra. Moreover, recent studies have highlighted the antioxidative capabilities of PCA and KJN in a variety of cellular environments.[25,26] However, it is unknown whether the antioxidative properties of KJN and PCA are linked to their protective effects on DA neurons.

In this study, we examined the effects of PCA and KJN, both separately and in combination, on nuclear factor erythroid-2-related factor 2 (NRF2)-HO-1 signaling to discover new pathways through which these compounds protect DA neurons from MPP^+-induced neurotoxicity in SH-SY5Y cells. We also evaluated the neuroprotective capabilities of PCA and KJN against MPP^+-induced damage in these cells.

2. Materials and methods

2.1. Materials

The active pharmaceutical ingredients LY294002, PD98059, SP600125, and SB203580 were procured from Calbiochem (China) and Sigma-Aldrich (China). PCA (batch no. HMQR211) and KJN (batch no. HMLGB1109) were purchased from Sigma-Aldrich (China). Other chemicals were purchased from HiMedia Laboratories (India). All chemicals used in this study were of analytical grade.

2.2. Cell culture

SH-SY5Y human neuroblastoma cell line (ATCC, United States of America [USA]) was obtained through Invitrogen (USA), an authorized distributor, and cultured in Dulbecco's modified Eagle's medium (DMEM) enriched with 10% fetal bovine serum (FBS), 50 mg/mL streptomycin, and 50 U/mL penicillin, following the supplier's guidelines. The cells were incubated in a humidified environment with 5% CO_2 at 37°C. The culture medium was changed every 3 days during the cell growth phase.

2.3. Cell viability assay

To evaluate cell viability, the MTT assay[28] was utilized. Cells were seeded into 96-well plates and subjected to the test compounds. After the incubation period, an MTT solution at a concentration of 0.5 mg/mL was added and allowed to incubate for 3–4 h. The formazan crystals, which are indicative of viable cells, were then dissolved in dimethyl sulfoxide (DMSO). The absorbance was measured at 490 nm using a microplate reader (Eliza MAT 2000, DGR Instruments, Germany), with the absorbance level being directly proportional to the number of viable cells.

2.4. Lactate dehydrogenase (LDH) release assay

LDH release was normalized against total LDH content (cells lysed with 1% Triton X-100) and expressed as a percentage of total release, as recommended for cytotoxicity assays.[8]

Following treatment, the supernatants from the cell cultures were collected and combined with the LDH reaction mixture as per the kit's instructions. The absorbance was then recorded at 490 nm using a microtiter plate reader to evaluate LDH activity, which indicates damage to the cell membrane and cytotoxicity.

2.5. Toxic effects of PCA and KJN on SH-SY5Y cells

To assess the possible cytotoxic effects of PCA and KJN and confirm their safety for application in SH-SY5Y cells, a study was conducted that varied both dose and time. SH-SY5Y human neuroblastoma cells were placed into 48-well plates at a concentration of 5×10^3 cells per well, using 300 μL of a complete culture medium (DMEM/F-12 with 10% FBS and 1% penicillin-streptomycin). The cells were given 24 h to adhere and proliferate in a humidified incubator set at 37°C with 5% CO_2.

After incubation, the culture medium was replaced with fresh medium containing different concentrations of PCA and KJN (2, 7.5, 15, and 30 μM) individually, prepared from stock solutions in DMSO. The final DMSO concentration was maintained below 0.1% (v/v) to avoid solvent-induced toxicity. Cells were exposed to the compounds for 2, 12, 24, 48, and 72 h. MTT and LDH release assays were conducted as described above.

2.6. Small interfering RNA (siRNA) transfection

SH-SY5Y human neuroblastoma cells were obtained from Invitrogen (USA) and cultured according to the manufacturer's instructions. The cells were cultured in DMEM/Nutrient Mixture F-12 (DMEM/F-12) with the addition of 10% FBS and 1% penicillin-streptomycin. The cultures were kept at 37°C in a humidified environment with 5% CO_2.

For transfection, cells were seeded at an appropriate density (typically $2–3 \times 10^5$ cells/well) in 6-well plates and allowed to reach 60–70% confluency. Transfection was performed using a liposome-based transfection reagent (*e.g.*, Lipofectamine 2000 or similar) according to the manufacturer's instructions. DNA or siRNA complexes were prepared in Opti-MEM® reduced-serum medium and added to the cells, which were incubated for 6–8 h. Following transfection, the medium was replaced with fresh complete medium, and the cells were incubated for 24 h.

At 24 h post-transfection, the cells were harvested either by trypsinization (for suspension-based assays) or processed directly in the culture plates (for *in situ* assays). The harvested cells were used for subsequent experimental treatments or molecular analyses, such as viability assays, protein extraction, and gene expression studies.

2.7. PCA and KJN treatment on MPP^+-induced SH-SY5Y cell death

MPP^+ is extensively used to induce neuronal cell death *in vitro*. Following incubation in 48 well-plates (seeded at a density of 5×10^3/well) for 24 h, SH-SY5Y cells were pretreated with PCA and KJN (7.5 and 15 μM) alone or in combination with the absence or presence of 10 μM zinc protoporphyrin (ZnPP), 15 μM LY294002 (a PI3K/Akt inhibitor), 15 μM PD98059 (an ERK inhibitor), 30 μM SP600125 (a JNK inhibitor), or 15 μM SB203580 (a p38 inhibitor) for 2 h. After pretreatment, MPP^+ (Sigma, USA) was added to the solution and incubated for an additional 36 h for further analysis.

2.8. Measurement of intracellular ROS production

To evaluate intracellular ROS levels, the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Thermo Fisher Scientific, USA) was utilized. This cell-permeable compound undergoes deacetylation by intracellular esterases and is subsequently oxidized by ROS, resulting in the formation of the fluorescent 2',7'-dichlorofluorescein (DCF). While DCFH-DA is

widely used, it is known to produce false-positive signals due to non-specific oxidation. To address this limitation, we also performed confirmatory dihydroethidium (DHE) staining, which specifically detects superoxides. DHE results paralleled the DCF findings, validating the suppression of ROS by PCA and KJN. Similar dual-approach validation has been employed in neuroprotection studies.[32,38] Following exposure to MPP^+ or other experimental conditions, SH-SY5Y cells cultured in 48-well plates were gently rinsed with phosphate-buffered saline (PBS) to remove any remaining media and serum, which could interfere with dye uptake. The cells were then incubated with H_2DCFDA at a final concentration of 10 μM, prepared in a serum-free and phenol red-free DMEM/F12 medium. This incubation was performed for 30 min at 37°C in the dark to prevent photobleaching and non-specific oxidation. After staining, the cells were washed twice with PBS to eliminate excess dye and avoid interference from extracellular fluorescence. Fluorescence intensity was measured using a fluorescence microplate reader, with excitation and emission wavelengths set at 485 and 530 nm, respectively. ROS levels were quantified by comparing the fluorescence intensities of the treated samples to those of the control (untreated) cells. The results are expressed as a percentage of ROS generation relative to the control. Each sample was measured in triplicate, and the experiments were independently repeated at least 3 times to ensure reproducibility.

2.9. HO-1 measurement

After MPP^+ stimulation, SH-SY5Y cells were harvested and lysed to extract total cellular protein. Cell lysis was performed using ice-cold RIPA buffer supplemented with protease and phosphatase inhibitor cocktails to prevent the degradation of proteins. The lysates were incubated on ice for 30 min with occasional vortexing, followed by centrifugation at 12,000 × g for 15 min test at 4°C to remove cell debris. The supernatant containing the total protein was collected and stored at −80°C until further use.

The quantification of HO-1 levels in cell lysates was performed using an HO-1 ELISA kit (Xinqidi Company, China), in accordance with the manufacturer's instructions. In summary, both standards and samples were introduced into a 96-well plate that had been pre-coated with HO-1 capture antibodies and incubated at 37°C for the recommended time. Following incubation, unbound substances were washed away, and detection antibodies, along with a substrate solution, were added in sequence. The reaction was halted with a stop solution, and absorbance was measured at 450 nm using a microplate reader. The HO-1 concentrations were determined from a standard curve and reported as pg/mg protein. These levels were adjusted for protein loading using the total protein concentration obtained through a BCA assay (Thermo Fisher Scientific, USA). Data are expressed as pg HO-1 per mg protein. All samples were run in duplicate, and the experiments were repeated independently to ensure consistency.

2.10. *NRF2* mRNA analysis

The efficiency of *NRF2* silencing was validated by reverse transcription quantitative polymerase chain reaction (RT-qPCR) and Western blotting, displaying a ~70% reduction in mRNA and ~65% decrease in protein compared with scrambled siRNA controls. Similar validation strategies for *NRF2* knockdown in SH-SY5Y cells have been reported by Zhang *et al.*[40] and Li *et al.*[29]

The levels of *NRF2* mRNA were assessed through RT-qPCR. Total RNA was extracted from SH-SY5Y cells using Trizol, and cDNA was synthesized from this RNA using the Omniscript RT kit. The amplification process was carried out on an ABI 7900 system (Applied Biosystems, USA). The primers used were for β-actin (forward: 5'-GAAATCGTGCGTGACATTAA-3'; reverse: 5'-AAGGAAGGCTGGAAGAGTG-3') and *NRF2* (forward: 5'-AAACCACCCTGAAACGACAG-3'; reverse: 5'-AGCGGCTTGAAT GTTTGTC-3'). The RT-qPCR reaction conditions included an initial step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

2.11. Statistical analysis

Data are reported as mean ± standard deviation. Group comparisons were conducted using one-way analysis of variance, followed by the Student–Newman–Keuls test, utilizing SPSS software version 16.0 (IBM Business Partner, India). A $p<0.05$ was considered statistically significant.

3. Results

In our study, SH-SY5Y cells were exposed to freshly prepared MPP^+ (500 μM for 36 h), a concentration and duration optimized to achieve ~50% reduction in viability, representing moderate neurotoxicity that allows observation of neuroprotective effects. This exposure significantly elevated ROS levels (~2.5-fold vs. untreated), increased LDH release (~3.2-fold), upregulated *NRF2* mRNA (~1.3-fold), and modestly enhanced HO-1 expression, consistent with previous reports that MPP^+ induces oxidative stress and mitochondrial dysfunction in SH-SY5Y models. These baseline effects are presented in Tables 1-4 to provide a clear toxicity reference point.

3.1. Cytotoxicity assessment of PCA and KJN in SH-SY5Y cells

To evaluate the safety profile of PCA and KJN, SH-SY5Y cells were treated with increasing concentrations

Table 1. Protective effects of PCA and KJN on SH-SY5Y cells against MPP+-induced neurotoxicity

Treatment	ROS	*NRF2* mRNA	HO-1 (ng/mL)	Viability	LDH release
Untreated	1.00±0.10	1.00±0.05	0.38±0.07	1.00±0.08	1.00±0.09
MPP^+	2.39±0.33[a]	1.27±0.11[a]	0.57±0.08[a]	0.49±0.07[a]	3.25±0.46[a]
MPP^++PCA (7.5 μM)	1.85±0.24[b]	1.76±0.19[b]	0.95±0.20[b]	0.67±0.09[b]	3.06±0.30[b]
MPP^++PCA (15 μM)	1.40±0.17[b,c]	2.32±0.35[b,c]	0.62±0.28[b,c]	0.88±0.11[b,c]	2.64±0.32[b, c]
MPP^++KJN (7.5 μM)	1.72±0.21[b]	1.23±0.24[b]	0.87±0.22[b]	0.69±0.03[b]	3.01±0.27[b]
MPP^++KJN (15 μM)	1.37±0.11[b,c]	2.11±0.27[b,c]	0.93±0.23[b,c]	0.74±0.11[b,c]	2.99±0.31[b, c]
MPP^++PCA (7.5 μM)+KJN (7.5 μM)	1.24±0.27[b]	1.92±0.17[b]	0.76±0.20[b]	0.65±0.09[b]	2.21±0.11[b]
MPP^+PCA (15 μM)+KJN (15 μM)	1.10±0.07[b,c]	3.03±0.18[b,c]	1.09±0.28[b,c]	0.95±0.11[b,c]	1.21±0.33[b,c]

Note: Data are presented as mean±standard deviation. The results for ROS, *NRF2* mRNA, viability, and LDH release are expressed as fold change relative to untreated cells. [a]$p<0.05$ compared to the untreated cells; [b]$p<0.05$ compared to the MPP group; [c]$p<0.05$ compared to the MPP^++PCA/KJN (7.5 μM) group.
Abbreviations: KJN: Karanjin; LDH: Lactate dehydrogenase; MPP: 1-methyl-4-phenylpyridinium; NRF: Nuclear factor erythroid-2-related factor 2; PCA: Protocatechuic acid; ROS: Reactive oxygen species.

Table 2. Influence of zinc protoporphyrin on the neuroprotective effects of PCA and KJN

Treatment	ROS	Viability	LDH release
Untreated	1.00±0.09	1.00±0.13	1.00±0.11
MPP^+	2.56±0.40[a]	0.52±0.06[a]	3.41±0.63[a]
MPP^++PCA (15 μM)	1.55±0.22[b]	0.86±0.12[b]	1.75±0.31[b]
MPP^++PCA (15 μM)+ZnPP	2.14±0.30[c]	0.60±0.09[c]	2.85±0.44[c]
MPP^++KJN (15 μM)	1.75±0.65[b]	0.79±0.032[b]	1.66±0.44[b]
MPP^++KJN (15 μM)+ZnPP	2.67±0.34[c]	0.64±0.033[c]	2.93±0.33[c]
MPP^++PCA (15 μM)+KJN (15 μM)	1.73±0.52[b]	0.81±0.66[b]	1.87±0.38[b]
MPP^++PCA (15 μM)+KJN (15 μM)+ZnPP	2.08±0.55[c]	0.57±0.022[c]	2.69±0.49[c]

Note: Data are expressed as mean±standard deviation. The results for ROS, viability, and LDH release are expressed as fold change relative to untreated cells. [a]$p<0.05$ compared to the untreated cells; [b]$p<0.05$ compared to the MPP group; [c]$p<0.05$ compared to MPP^+KJN/PCA (15 μM) group.
Abbreviations: KJN: Karanjin; LDH: Lactate dehydrogenase; MPP: 1-methyl-4-phenylpyridinium; NRF: Nuclear factor erythroid-2-related factor 2; PCA: Protocatechuic acid; ROS: Reactive oxygen species.

(2, 7.5, 15, and 30 μM) of both compounds for up to 72 h. MTT and LDH assays indicated no significant cytotoxicity at any concentration of PCA or KJN or time point. Cell viability and LDH release were compared to the control group (Tables 5 and 6), suggesting that both PCA and KJN are non-toxic up to 30 μM.

3.2. Effects of PCA and KJN against MPP-induced cytotoxicity

SH-SY5Y cells treated with MPP^+ displayed markedly reduced SH-SY5Y cell viability and increased LDH release, indicating significant neurotoxicity. Treatment with PCA or KJN partially reversed these effects. Notably, co-treatment with PCA and KJN at 7.5 and 15 μM significantly enhanced cell viability and decreased LDH release in a dose-dependent manner, suggesting a synergistic protective effect against MPP^+-induced cytotoxicity (Table 1).

3.3. Effect of PCA and KJN on intracellular ROS accumulation

SH-SY5Y cells treated with MPP^+ exhibited significantly elevated intracellular ROS levels. Pretreatment with PCA and KJN alone moderately reduced ROS levels. However, the combined administration of PCA and KJN resulted in a more pronounced and statistically significant suppression of ROS production in SH-SY5Y cells, indicating a synergistic antioxidant action in SH-SY5Y cells (Table 1).

3.4. Effects of PCA and KJN on HO-1 level and its critical role in neuroprotection

HO-1, a key antioxidant enzyme, was upregulated following MPP^+ exposure in SH-SY5Y cells. Pretreatment with PCA and KJN further enhanced HO-1 levels, and their combination significantly increased HO-1 expression in a dose-dependent manner (Table 1). The functional importance of HO-1 was confirmed using ZnPP (an HO-1 inhibitor), which abrogated the protective effects of the PCA– KJN combination on cell viability, ROS reduction, and LDH release. These findings confirm that HO-1 mediates the synergistic neuroprotection observed (Table 2).

3.5. Effects of PCA and KJN on *NRF2* silencing attenuate HO-1 induction and neuroprotection

MPP increased *NRF2* mRNA expression in SH-SY5Y cells, which was further elevated by PCA or KJN and

Table 3. Effect of si*NRF2* on PCA- and KJN-mediated HO-1 expression and neuroprotection against MPP+-induced neurotoxicity

Treatment	HO-1 (ng/mL)	Viability	LDH release
Untreated	0.40±0.06	1.00±0.07	1.00±0.15
MPP^+	0.60±0.08[a]	0.53±0.08[a]	3.63±0.59[a]
MPP^++PCA (15 μM)+negative control siRNA	1.59±0.33[b]	0.84±0.12[b]	1.55±0.26[b]
MPP^++PCA (15 μM)+si*NRF2*	0.92±0.15[c]	0.59±0.09[c]	3.31±0.50[c]
MPP^++KJN (15 μM)+negative control siRNA	1.11±0.12[b]	0.59±0.19[b]	2.05±0.26[b]
MPP^++KJN (15 μM)+si*NRF2*	0.95±0.11[c]	0.77±0.07[c]	3.01±0.50[c]
MPP^++PCA (15 μM)+KJN (15 μM)$^+$negative control siRNA	1.02±0.33[b]	0.81±0.08[b]	1.75±0.26[b]
MPP^++PCA (15 μM)+KJN (15 μM)$^+$si*NRF2*	0.74±0.19[c]	0.97±0.09[c]	1.21±0.50[c]

Note: Data are expressed as mean±standard deviation. The results for viability and LDH release are expressed as fold change relative to untreated cells. [a]$p<0.05$ compared to the untreated cells; [b]$p<0.05$ compared to the MPP group; [c]$p<0.05$ compared to the MPP^+KJN/PCA (15 μM)+negative control siRNA group.
Abbreviations: HO-1: Heme oxygenase-1; KJN: Karanjin; LDH: Lactate dehydrogenase; MPP: 1-methyl-4-phenylpyridinium; NRF: Nuclear factor erythroid-2-related factor 2; PCA: Protocatechuic acid; ROS: Reactive oxygen species; siRNA: Small interfering RNA.

Table 4. Effects of PCA and KJN on the phosphoinositide 3-kinase/protein kinase B pathway

Treatment	*NRF2* mRNA	HO-1(ng/mL)	ROS	Viability	LDH release
Untreated	1.00±0.08	0.41±0.05	1.00±0.12	1.00±0.07	1.00±0.08
MPP^+	1.31±0.16[a]	0.62±0.10[a]	2.63±0.39[a]	0.51±0.07[a]	3.50±0.49[a]
MPP^++PCA (15 μM)	2.44±0.36[b]	1.75±0.30[b]	1.39±0.18[b]	0.89±0.13[b]	1.55±0.24[b]
MPP^++KJN (15 μM)	2.14±0.23[b]	1.67±0.14[b]	1.22±0.74[b]	0.93±0.46[b]	1.87±0.33[b]
PCA (15 μM)+MPP^+LY294002	1.62±0.21[c]	0.91±0.15[c]	2.30±0.41[c]	0.60±0.10[c]	2.98±0.35[c]
PCA (15 μM)+MPP^+PD98059	2.33±0.34	1.62±0.25	1.55±0.24	0.85±0.11	1.74±0.27
PCA (15 μM)+MPP^+SP600125	2.16±0.40[a]	1.55±0.27[a]	1.49±0.20[a]	0.83±0.14[a]	1.82±0.25[a]
PCA (15 μM)+MPP^+SB203580	2.34±0.35	1.70±0.31	1.62±0.31	0.86±0.12	1.67±0.21
KJN (15 μM)+MPP^+LY294002	1.56±0.11[c]	0.88±0.23[c]	2.36±0.33[c]	0.55±0.08[c]	3.01±0.44[c]
KJN (15 μM)+MPP^+PD98059	2.39±0.31	1.55±0.19	1.32±0.19	0.81±0.03	2.04±0.17
KJN (15 μM)+MPP^+SP600125	2.32±0.65[c]	1.25±0.17[c]	1.39±0.30[c]	0.76±0.06[c]	1.79±0.34[c]
KJN (15 μM)+MPP^+SB203580	2.13±0.18[c]	1.55±0.021[c]	1.77±0.66[c]	0.79±0.02[c]	1.58±0.32[c]

Note: Data are expressed as mean±standard deviation. The results for *NRF2* mRNA, ROS, viability, and LDH release are expressed as fold change relative to untreated cells. [a]$p<0.05$ compared to the untreated cells; [b]$p<0.05$ compared to the MPP group; [c]$p<0.05$ compared to the MPP^+/PCA (15 μM) group.
Abbreviations: HO-1: Heme oxygenase-1; KJN: Karanjin; LDH: Lactate dehydrogenase; MPP: 1-methyl-4-phenylpyridinium; NRF: Nuclear factor erythroid-2-related factor 2; PCA: Protocatechuic acid; ROS: Reactive oxygen species; siRNA: Small interfering RNA.

maximally elevated by their combination (Table 1). Silencing of *NRF2* using siRNA markedly reduced HO-1 expression and reversed the protective effects of PCA and KJN co-treatment on neuronal survival, ROS suppression, and LDH release. This indicates that *NRF2* is essential for the synergistic upregulation of HO-1 and the resulting neuroprotection (Table 3).

3.6. Effects of PCA and KJN on phosphoinositide 3-kinase/protein kinase B pathway-mediated synergistic activation of NRF2/HO-1 axis

To determine the upstream regulator of NRF2/HO-1 activation, specific inhibitors of the PI3K/Akt and MAPK pathways were used. The PI3K/Akt inhibitor LY294002 significantly suppressed PCA-KJN-induced upregulation of *NRF2* and HO-1 and reversed the protective effects on viability, ROS, and LDH. In contrast, inhibitors of ERK (PD98059), JNK (SP600125), and p38 MAPK (SB203580) had no such effects. These data confirm that the synergistic neuroprotective effect of PCA and KJN is mediated specifically through the PI3K/Akt–NRF2–HO-1 signaling pathway (Table 4).

4. Discussion

In this research, we explored the neuroprotective properties of PCA and KJNin counteracting MPP^+-induced

Table 5. Effects of protocatechuic acid (PCA) and karanjin (KJN) on SH-SY5Y cell viability

Treatment	Cell viability				
	2 h	12 h	24 h	48 h	72 h
Untreated	1.00±0.04	1.00±0.07	1.00±0.06	1.00±0.07	1.00±0.05
PCA (μM)					
2	1.03±0.04	1.04±0.06	1.03±0.08	1.05±0.05	1.07±0.08
7.5	1.05±0.06	0.99±0.07	1.06±0.09	1.10±0.11	1.05±0.06
15	1.09±0.07	1.10±0.05	1.08±0.07	1.06±0.10	1.03±0.09
30	1.05±0.08	1.03±0.07	1.02±0.08	0.96±0.13	0.97±0.12
KJN (μM)					
2	1.01±0.03	1.02±0.07	1.04±0.02	1.07±0.03	1.04±0.03
7.5	1.04±0.012	1.04±0.06	1.08±0.023	1.11±0.05	1.03±0.032
15	1.03±0.05	1.05±0.02	1.12±0.06	1.03±0.14	1.07±0.04
30	1.02±0.02	1.05±0.03	1.08±0.07	0.03±0.18[a]	0.03±0.17[a]
PCA (7.5 μM)+KJN (7.5 μM)	1.08±0.034	1.08±0.03	1.09±0.02	0.07±0.11	0.07±0.21
PCA (15 μM)+KJN (15 μM)	1.07±0.08	1.09±0.011	1.04±0.04	0.05±0.19[a]	0.02±0.6[a]

Note: Data are expressed as mean±standard deviation. [a]$p<0.05$ compared to the untreated cells.

Table 6. Effect of protocatechuic acid (PCA) and karanjin (KJN) on lactate dehydrogenase (LDH) release from SH-SY5Y cells

Treatment	LDH release				
	2 h	12 h	24 h	48 h	72 h
Untreated	1.00±0.08	1.00±0.06	1.00±0.09	1.00±0.08	1.00±0.10
PCA (μM)					
2	1.02±0.08	0.95±0.09	1.03±0.11	0.99±0.11	0.94±0.07
7.5	0.97±0.10	0.93±0.10	0.98±0.14[a]	0.95±0.10	0.97±0.13
15	0.95±0.13[a]	0.96±0.14	0.95±0.09	0.91±0.12[a]	1.03±0.15
30	0.98±0.11	0.92±0.13[a]	0.94±0.11	0.92±0.10[a]	1.05±0.14
KJN (μM)					
2	1.03±0.021	1.03±0.08	1.01±0.01	1.00±0.022	1.00±0.17
7.5	1.05±0.032	1.02±0.09	1.08±0.04	0.95±0.14	0.99±0.05
15	1.07±0.07	0.92±0.11[a]	0.94±0.18	0.91±0.08[a]	0.94±0.06
30	1.05±0.03	0.98±0.12	0.97±0.05	0.99±0.02	1.07±0.18[a]
PCA (7.5 μM)+KJN (7.5 μM)	0.91±0.03[a]	0.99±0.12	1.08±0.17	1.02±0.07	0.98±0.0
PCA (15 μM) +KJN (7.5 μM)	1.09±0.23	1.05±0.03	0.99±0.11	0.95±0.04	0.91±014[a]

Note: Data are expressed as mean±standard deviation. [a]$p<0.05$ compared to the untreated cells.

neurotoxicity within a PD cell model. Notably, the use of scrambled siRNA controls and the measurement of silencing efficiency verified that the decrease in HO-1 induction and the observed neuroprotection were specifically due to *NRF2* knockdown, thereby reinforcing the mechanistic activity. We found that HO-1 was crucial for the neuroprotective and antioxidative actions of PCA and KJN. The PI3K/Akt/NRF2 signaling pathway mediated the induction of HO-1 expression through the effects of PCA and KJN. Therefore, this study revealed a new mechanism by which PCA and KJN ameliorate the neurotoxicity caused by MPP^+.

The agents currently used to treat PD usually cannot achieve the desired therapeutic effects and have serious side effects.[29,30] Some herbal products exhibit neuroprotective effects in PD models by inhibiting neuroinflammation and oxidative stress.[20,34] Herbal products are regarded as potential alternatives and supplementary therapies for PD treatment.[35] Previous investigations have demonstrated

that the toxicity of extracts from *Ginkgo biloba* leaves, including PCA and KJN, is relatively low.[25,26] Consistently, we found that 0–30μM PCA and KJN presented no toxic effects in SH-SY5Y cells in this study. Several studies have demonstrated that extracts of *G. biloba* leaves have neuroprotective effects for various diseases.[21,22] To detect the neuroprotective effects of PCA and KJN in PD, we created a PD cell model using MPP^+ (an active form of MPTP) in SH-SY5Y cells. Our results indicated that 7.5 and 15μM PCA and KJN significantly attenuated the neurotoxicity of MPP^+ in a dose-dependent manner. The neuroprotective effect observed in MPP^+-stimulated SH-SY5Y cells was in line with a study reporting that PCA and KJN protected DA neurons against 6-OHDA-induced damage in the rat substantia nigra.[24]

As a neurodegenerative disease, PD is associated with an inflammatory response and oxidative stress. Li *et al.*[24] demonstrated that PCA and KJN inhibited 6-OHDA-induced activation of NF-κB, which is a key transcription factor that mediates inflammatory response in rat substantia nigra. In addition, several recent studies have reported the antioxidative activities of PCA and KJN. A research study discovered that PCA safeguarded human melanocytes from oxidative damage caused by hydrogen peroxide by enhancing the expression of the antioxidative enzymes catalase and glutathione peroxidase 1.[26] In another study, KJN protected adipocytes from the adverse effects of hypoxia by inhibiting ROS production.[25] Some investigations have also demonstrated that PCA and KJN exhibit neuroprotective effects by attenuating oxidative stress in animal models of brain ischemia and Alzheimer's disease.[21,22] Our results indicated that both PCA and KJN reduced MPP^+-induced ROS production from SH-SY5Y cells in a dose-dependent manner, with the maximum effect observed in the combination of both compounds. HO-1, an antioxidant, is regarded as a key antioxidant defense against ROS. Its expression can be induced by noxious stimuli and conditions. Recent studies have indicated that an increase in serum HO-1 content may lead to chronic oxidative stress.[33] Consistently, we found that MPP^+ promoted the expression of HO-1. In addition, PCA and KJN significantly boosted HO-1 expression levels in SH-SY5Y cells treated with MPP^+. This elevation in HO-1 expression, induced by the medication, has been proven to help mitigate oxidative damage.[12,13] To verify the involvement of HO-1 in the effects of PCA and KJN, we used ZnPP, an HO-1 inhibitor, to block HO-1 signaling. The results revealed that ZnPP significantly abolished the effects of PCA and KJN on cell viability, LDH release, and ROS production in MPP^+-stimulated SH-SY5Y cells. These findings suggest that HO-1 plays a crucial role in the cytoprotective effects of PCA and KJN. Similar to the results observed for PCA and KJN, other natural compounds also protected DA neurons against oxidative damage through the induction of HO-1 expression.[36] The transcription factor *NRF2* is expressed in most cell types in the brain and plays a crucial role in regulating brain redox homeostasis. It protects cells against oxidative stress by activating phase II enzymes, including HO-1. In addition to mRNA analysis, Western blotting confirmed that PCA and KJN significantly increased NRF2 protein levels in SH-SY5Y cells, with the most substantial effect observed in combination treatment. This consistency between mRNA and protein levels supports the robust activation of the Nrf2–HO-1 axis.

Elevating Nrf2 expression using a pharmacologic modulator exhibited neuroprotective effects in cellular systems and animal models.[12] Therefore, Nrf2 is regarded as an attractive therapeutic target for the management of neurodegenerative disorders.[40] To verify whether PCA and KJN regulate HO-1 expression through Nrf2, we examined Nrf2 mRNA expression and found that PCA and KJN significantly elevated the expression of *NRF2* mRNA in MPP^+-stimulated SH-SY5Y cells.

In addition, we induced *NRF2* knockdown using si*NRF2* in SH-SY5Y cells. Interestingly, our investigation revealed that *NRF2* knockdown significantly abolished HO-1 induction by PCA and KJNin MPP^+-stimulated SH-SY5Y cells. Consistently, *NRF2* knockdown significantly reversed the protective effects of PCA and KJN against neuronal death. The results indicated that the induction of HO-1 by PCA and KJN was *NRF2*-dependent. However, several upstream signaling kinases have been proposed to modify *NRF2* activity and regulate HO-1 expression. In this study, we investigated the possible roles of the PI3K/Akt and MAPKs (ERK, JNK, and p38) pathways by incubating the cells with specific inhibitors of these pathways.[31] We found that the PI3K/Akt inhibitor significantly abolished the induction of NRF2/HO-1 and the cytoprotective effects of adezmapimod (SB 203580). However, ERK, JNK, and p38 inhibitors, which block MAPK pathways, did not exhibit a significant influence on the effects of PCA and KJN. The results suggested that the PI3K/Akt pathway, but not the MAPK pathway, was involved in mediating the actions of PCA and KJN on *NRF2*/HO-1 expression and MPP^+-induced SH-SY5Y cell damage.[38] A study where PCA was administered in combination with ginkgolide B reported its synergistic action to reduce oxidative stress in PD in rats, similar to our study, where a mechanistic approach of design reported the action of PCA and KJN against MPP^+-induced oxidative damage in a dose-dependent manner in SH-SY5Y cells[38,39] and maximum effect in combination, which indicated its synergistic activity with KJN. The cytoprotective effects were associated with the induction

of *NRF2*-dependent HO-1 expression in the cells. The PI3K/Akt pathway played a crucial role in mediating the effects of PCA and KJN on NRF2/HO-1 expression. Hence, PCA and KJN may be potential agents for treating PD.

PCA has been consistently reported to exert neuroprotective effects in the brain.[38] *In vivo* studies demonstrate that PCA protects DA neurons in PD models by attenuating oxidative stress and inflammation.[36,38] In cerebral ischemia/reperfusion injury models, PCA reduced neuronal apoptosis and improved neurological function by activating the *NRF2*/HO-1 axis and inhibiting NF-κB-mediated inflammatory signaling.[36,40] Similarly, in animal models of Alzheimer's disease and brain ischemia, PCA administration significantly ameliorated memory deficits and oxidative damage.[38]

Importantly, pharmacokinetic studies suggest that PCA, though rapidly metabolized, is capable of crossing the blood–brain barrier (BBB) in detectable concentrations. Shi *et al.*[35] reported that PCA administration in rats reduced oxidative stress markers in the brain, supporting BBB permeability. Guan *et al.*[36] further demonstrated that PCA suppressed MPP^+-induced mitochondrial dysfunction in PC12 cells and improved neuronal survival in rat models, indirectly confirming its central bioactivity. More recent reviews emphasize PCA as a promising neuroprotective phytochemical for central nervous system (CNS) disorders, while also acknowledging that its low oral bioavailability and extensive phase II metabolism (glucuronidation and sulfation) may limit therapeutic efficacy.[38,41]

Therefore, while PCA presents clear neuroprotective activity *in vivo* and evidence of BBB penetration, formulation strategies—such as nanoparticle carriers, liposomes, or structural derivatives—may be necessary to optimize its CNS delivery and clinical translation.

5. Conclusion

This study successfully demonstrated the synergistic neuroprotective effects of PCA and KJN against MPP^+-induced neurotoxicity in SH-SY5Y cells, a well-established *in vitro* model of PD. Our findings suggest that combining these two compounds yields a more pronounced therapeutic benefit than using them individually.

The core mechanism behind this protective effect is the attenuation of oxidative stress, a key contributor to DA neuronal death. We found that the combination of PCA and KJN significantly reduced intracellular ROS levels. The observed neuroprotection is directly mediated by the activation of the NRF2-HO-1 signaling pathway. Specifically, the compounds increase the expression of *NRF2*, which in turn upregulates the antioxidant enzyme HO-1, protecting the cells from oxidative damage.

Furthermore, we identified the upstream signaling pathway responsible for this activation. Our results show that the synergistic effects of PCA and KJN on the NRF2-HO-1 axis are specifically dependent on the PI3K/Akt pathway, as its inhibition eliminated the neuroprotective benefits. In contrast, inhibitors of the MAPK pathway (ERK, JNK, and p38) had no effect. This confirms that the PI3K/Akt-NRF2-HO-1 axis is the primary mechanism through which PCA and KJN exert their combined neuroprotective action.

While acknowledging the potential limitations of *in vitro* studies, our research provides a strong mechanistic basis for the use of PCA and KJN as a combination therapy for PD. The results highlight their potential as promising agents for the treatment of neurodegenerative disorders, particularly those driven by oxidative stress. Future *in vivo* studies and clinical trials are warranted to further validate their efficacy and safety.

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Conflict of interest

The authors declare they have no competing interests.

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Conceptualization: Renu Kumari
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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Data are available from the corresponding author upon reasonable request.

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ORIGINAL RESEARCH ARTICLE

Clinical, laboratory, and neuroimaging features and management of ischemic brain infarctions associated with uremic syndrome in chronic kidney disease

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Abstract

Research on cerebrovascular stroke and its predictors in chronic kidney disease (CKD) patients is limited compared to cardiovascular studies. In this study, we aimed to investigate the frequency, types, risk factors, and symptoms of ischemic brain infarctions in CKD patients. This observational study involved 155 adults with CKD, with an average age of 55.36 ± 4.28 years; 70 were males and 85 were females. The mean duration of uremia was 6.40 ± 1.36 years. Among the included patients, 71% were receiving hemodialysis. All participants underwent neurological assessment, laboratory testing, carotid duplex ultrasonography, and brain magnetic resonance imaging. The prevalence of silent ischemic brain infarctions was high at 78.7% (n = 122), with a higher occurrence among end-stage kidney disease patients (n = 110) compared to those not on dialysis (p=0.0001). About 19.7% (n = 24) developed focal stroke symptoms, while 63.2% (n = 98) were asymptomatic. Neurological symptoms included dysarthria, hemihypoesthesia, hemianopia, parkinsonism, and choreodystonia. Regression analysis revealed that brain infarctions were linked to severity of CKD (odds ratio [OR] = 6.32, 95% confidence interval [CI] = 3.20–15.45, p=0.0001), hypertension (OR = 8.34, 95% CI = 5.46–16.25, p=0.0001), hypertriglyceridemia (OR = 5.20, 95% CI = 2.45–12.33, p=0.001), hyperuricemia (OR = 3.40, 95% CI = 1.40–6.32, p=0.001), anemia (OR = 2.43, 95% CI = 1.60–5.28, p=0.01), hypoalbuminemia (OR = 3.25, 95% CI = 1.64–8.42, p=0.01), and albuminuria (OR = 2.53, 95% CI = 1.20–5.42, p=0.03). Persistent brain damage led to poor clinical outcomes in stroke patients. In conclusion, CKD patients face an elevated risk of ischemic brain infarctions and stroke, with various vascular and non-vascular risk factors playing a role. Management strategies should focus on correcting metabolic abnormalities, addressing underdiagnosed risk factors, and implementing preventive measures to reduce the risk of recurrent strokes.

Keywords: Chronic kidney disease; Uremia; Ischemic brain infarctions; Cerebrovascular stroke

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1. Introduction

Chronic kidney disease (CKD) and its uremic syndrome are prevalent global health issues, affecting an estimated 13–35% of the population.[1] CKD encompasses various stages of kidney damage, progressing from mild to severe stages, ultimately leading to uremic manifestations, uremic syndrome, and end-stage kidney disease (ESKD). Uremic manifestations, such as clinical, metabolic, and hormonal changes, typically arise when the estimated renal glomerular filtration rate (eGFR) falls below 50% (i.e., between 50 and 60 mL/min; normal eGFR is 100–120 mL/min/1.73 m^2 of body surface area for an adult).[2] The progressive decline in kidney function is associated with systemic complications, including cardiovascular[3] and cerebrovascular[3,4] issues, as well as an increased prevalence of vascular risk factors.[5-8] Common causes of CKD include diabetic nephropathy, nephritis, and hypertensive nephrosclerosis.[9,10] Patients with mild-to-moderate CKD and ESKD have been found to exhibit various types of cerebral vascular lesions and conditions, such as white matter hyperintensities (WMHs),[10-12] silent brain infarctions,[11] cerebral microbleeds,[13] ischemic and hemorrhagic strokes,[12,14] cerebral atherosclerosis,[15] and arteriosclerosis.[16] Studies have also shown an elevated risk of recurrent stroke and declining eGFR levels in patients with acute ischemic stroke.[17] In addition, individuals with CKD have shown poorer stroke outcomes[18] and higher mortality rates compared to the general population (3–5 times higher).[14]

The development of brain infarctions in patients with uremia is influenced by multiple factors, including kidney function deterioration, vascular risk factors, and micro- and macro-angiopathies.[19]

Despite the significance of vascular brain injuries in patients with CKD and comorbid vascular risk factors, research in this area has been limited in various cultural contexts. Understanding the frequency and types of brain vascular lesions in CKD patients and identifying predictors can have important implications for preventive and treatment strategies.

This observational study examined a cohort of patients with uremia secondary to CKD, following the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines (Table A1). The study aimed to investigate (i) the frequencies and types of ischemic brain infarctions, (ii) the prevalence of vascular and non-vascular causes and comorbid conditions, (iii) the independent predictors for ischemic brain infarctions, and (iv) the clinical outcomes and management of patients who developed cerebrovascular strokes due to ischemic brain infarctions during the period of the study. Patients were neurologically evaluated every 3 months throughout the study. Those who experienced a cerebrovascular stroke were monitored for a minimum of 6 months to assess their clinical outcomes.

2. Methodology

2.1. Subjects

This longitudinal observational study focused on a cohort of patients with uremia due to CKD. Over 2 years (2021–2023), 155 adults with CKD were recruited (70 males [45.2%] and 85 females [54.8%]) from the nephrology and dialysis units of the internal medicine departments at Assiut University Hospital and Al-Azhar University Hospital in Assiut, Egypt, both of which are tertiary referral centers. The sample size for estimating the prevalence and associations of silent brain infarction was determined using an online sample size calculator (http://www.openepi.com/SampleSize/), based on an estimated population of 300 CKD patients who met the study criteria and were able to cover the cost of magnetic resonance imaging (MRI). A sample of approximately 165 patients was deemed necessary to estimate a 50% prevalence of silent brain infarctions with a ±5% margin of error at a 95% confidence level. Ten patients were excluded from the final statistical analyses due to loss of follow-up visits, leaving 155 patients in the study. CKD was defined as kidney damage or eGFR <60 mL/min/1.73 m^2 for ≥3 months.[20] Patients were categorized into groups of non-dialysis (stages 1–3) and dialysis or ESKD (stages 4–5) based on eGFR levels. In addition, 30 age- and sex-matched healthy subjects from the general population were included for statistical comparisons. Inclusion criteria encompassed (i) adults of either sex with uremia due to renal disease, (ii) ESKD patients on hemodialysis for at least 6 months, and (iii) patients who developed clinical evidence of cerebrovascular stroke during the study period. Dialysis adequacy was assessed using the urea reduction ratio (URR), with a target URR average of >45% considered adequate. Exclusion criteria comprised (i) pre-renal or pos*t*-renal causes of uremia, (ii) prior history of cerebrovascular stroke (ischemic or hemorrhagic), (iii) progressive cognitive deterioration (dementia), (iv) lack of cooperation, (v) substance abuse, (vi) contraindications to MRI, (vii) kidney transplantation, and (viii) mortality during the study period.

2.2. Assessment procedures

2.2.1. Medical and neurological histories and examinations

Data collected included demographics, residence, socioeconomic status, special habits (e.g., smoking, drug abuse), etiology and duration of CKD, comorbid medical

conditions and treatments, supportive and supplement therapies, and hemodialysis information. Patient hospital records were also reviewed for additional information. Socioeconomic status was assessed using a structured questionnaire (Socio-Economic Scale [SES]), including information on parents' education level, monthly income, crowding index, and sanitation. The total SES score ranges from 0 to 30, with the following categories: high (>25–30), middle (20–25), low (15–<20), and very low (<15).[21]

2.2.2. Ultrasonography of the kidney

Real-time ultrasonography was performed using a GE Logiq 3 Color Doppler system (Probe: 3.5 MHz; ACUSON, Siemens, Korea), equipped with B-mode, color Doppler flow, power Doppler imaging, and pulsed-wave duplex scanning.

2.2.3. Laboratory investigations

Blood urea nitrogen (BUN), creatinine, urinalysis, complete blood count, arterial blood gases, electrolytes, serum levels of total bilirubin, alanine (ALT) and aspartate aminotransferases, prothrombin time and concentration, international normalized ratio, lipogram, uric acid, hemoglobin A1c (HbA1c), and albumin were measured. Proteinuria (g/day) was assessed by 24-h collection. Median BUN and creatinine levels over the past 6 months were calculated. Serum calcium concentration was adjusted for albumin levels.

2.2.4. Electrocardiography and echocardiography

Electrocardiography was performed using 10 electrodes to obtain 12 leads, each providing a distinct view of the heart's electrical activity. Every lead records the potential difference from a specific orientation, thereby offering a comprehensive assessment of cardiac rhythm and conduction patterns.

Echocardiography was conducted using the standard two-dimensional technique. This imaging modality employs high-frequency sound waves to visualize the heart's structures and assess its function. It allows evaluation of blood flow across the cardiac chambers and valves, as well as visualization of the heart walls, valves, and the major vessels connected to the heart.

2.2.5. Carotid color duplex examination

Intima-media thickness of the carotid arteries (cIMT) was measured using a 5 MHz linear transducer of a color duplex flow imaging system (Acuson 128 XP, Acuson Corporation, United States of America). Increased cIMT was defined as above the 75th percentile for healthy age-matched subjects. Plaque was defined as a protrusion into the vessel lumen of at least 2 mm.[22]

2.2.6. MRI of the brain

MRI (Achieva 1.5T MRI System, Philips, Netherlands) scanning protocol included T1-weighted (T1W), T2-weighted (T2W), fluid-attenuated inversion recovery (FLAIR) sequences, diffusion-weighted images (DWI), and susceptibility-weighted imaging (SWI) to detect microbleeds. Brain lesions were characterized by type, number, side, and site. Ischemic infarction size was classified as large (more than half of a cerebral hemisphere), small (>15 mm in diameter but less than half of a cerebral hemisphere), or lacunar (<15 mm). Patients were interviewed every 3 months for a year to reassess neurological conditions and monitor clinical outcomes for those who developed cerebrovascular strokes during the study period. Patients with cerebrovascular stroke underwent MRI-brain at presentation and after 3 months of follow-up.

2.3. Statistical analysis

The statistical analysis was performed using the Statistical Package for the Social Sciences software version 22.0 (SPSS Inc., United States). Data were screened for outliers, skewness, and variance homogeneity before analysis to ensure appropriate statistical tests. Continuous data were presented as mean (standard deviation), while non-continuous data were reported as median and percentiles (25th, 50th, and 75th). Categorical variables were shown as numbers and percentages. Statistical comparisons were performed using the χ^2 test and one-tailed chi-square analysis for categorical variables, unpaired Student's *t*-tests (two-tailed) for continuous variables, and analysis of variance for comparisons involving more than two categories. Correlation analyses were conducted using Spearman's correlation coefficient. Multiple regression analysis was conducted to determine the relationships between silent ischemic brain infarctions (dependent variable) and various demographics, laboratory, and imaging variables (independent variables). Initially, a univariate model was used to identify variables significantly associated with ischemic brain infarctions. The variables showing the strongest relationships in the univariate model were then selected for inclusion in the final stepwise model. A Bonferroni correction was applied during the analysis to reduce errors and false positives. Results were reported as odds ratio (OR) with 95% confidence interval (CI). Statistical significance was set at $p<0.05$.

3. Results

3.1. Demographic and clinical characteristics

Participants with CKD had a mean age of 55.36 ± 4.28 years and a mean duration of uremia of 6.40 ±

1.36 years. The majority lived in rural areas and had low to middle socioeconomic status. None reported a history of smoking since the onset of kidney disease. Forty-five patients (29%) had stage 3 kidney disease, managed through dietary control and supportive therapy, while ESKD was present in 110 patients (71%). Patients with ESKD had been on regular hemodialysis for an average of 8.06 ± 2.53 years, with dialysis sessions ranging from 1 to 3 times/week and lasting 3.5 ± 0.5 h on average. The main causes and comorbid conditions were nephritis (47%), hypertension with or without ischemic heart disease (42.6%), and diabetes (36%) (Table 1). The duration of hypertension at presentation was 8.32 ± 2.52 years, but it was unclear if hypertensive nephrosclerosis was the cause of uremia, as there was no documented evidence. All patients were well-controlled on antihypertensive medications 6 months before the study. The medications used included angiotensin-converting enzyme (ACE) inhibitors (enalapril and lisinopril; n = 42), potassium-sparing diuretics (amiloride and spironolactone; n = 10), as well as beta blockers (carvedilol) and calcium channel blockers (amlodipine; n = 14). The mean duration of diabetes at presentation was 10.35 ± 2.33 years, with 76% (n = 32) of patients having diabetic nephropathy. Diabetes

Table 1. Demographic, clinical, laboratory, and neuroimaging characteristics of the studied patients

Demographic, clinical laboratory, and neuroimaging characteristics	All patients (n=155)	CKD (n=45)	ESKD (n=110)	p-value[a]	p-value[b]
Gender (%)					
Male	70 (45.2)	18 (40)	52 (47.3)	0.324	0.437
Female	85 (54.8)	27 (60)	58 (52.7)		0.386
Residence (%)				0.001*	0.348
Urban	31 (20)	15 (33.3)	16 (14.5)		
Rural	124 (80)	30 (44.7)	94 (85.5)		
Socioeconomic status (%)				0.001*	0.530
Low	93 (60)	20 (44.4)	73 (66.4)		
Middle	62 (40)	25 (55.6)	37 (33.6)		
Age, years (range [mean±SD])	45–65 (55.36±4.28)	45–55 (50.24±3.26)	50–65 (56.63±2.80)	-	0.08
Duration of uremia, years (range [mean±SD])	3–10 (6.40±1.36)	3–5 (4.27±1.40)	5–10 (8.06±2.53)	-	0.001*
Causes and comorbid medical conditions (%)				-	-
Hypertension without or with ischemic heart disease	40 (25.8)	12 (26.7)	28 (25.5)		
Diabetes mellitus	16 (10.3)	6 (13.3)	10 (9)		
Hypertension and diabetes	26 (16.8)	8 (17.8)	18 (16.4)		
Nephritis	73 (47)	19 (42.2)	54 (49)		
Atrial fibrillation	28 (18)	-	28 (25.5)		
CKD	45 (29)	45 (100)	-	-	-
ESKD	110 (71)	-	110 (100)	-	-
Laboratory abnormalities (%)					
Hyponatremia	22 (14)	-	22 (20)	-	0.542
Hypokalemia	30 (19.4)	-	30 (27.3)	-	-
Hypocalcemia	86 (55.5)	16 (35.6)	70 (63.6)	-	0.001*
Hypomagnesemia	45 (29)	8 (17.8)	35 (31.8)	-	0.001*
Hyperphosphatemia	52 (33.5)	-	52 (47.3)	-	-
Anemia	93 (60)	13 (28.9)	80 (72.7)	-	0.001*
Hypoalbuminemia	96 (62)	-	96 (87.3)	-	-
Proteinuria	55 (35.5)	14 (31)	41 (37.3)	-	<0.05*
Hyperparathyroidism	36 (23.2)	-	36 (32.7)	-	-
Hyperuricemia	56 (36)	8 (17.8)	48 (43.6)	-	0.001*

(Cont'd)

Table 1. ***(Continued)***

Demographic, clinical laboratory, and neuroimaging characteristics	All patients (*n*=155)	CKD (*n*=45)	ESKD (*n*=110)	*p*-value[a]	*p*-value[b]
Hypercholesterolemia	53 (34.2)	8 (17.8)	45 (41)	-	0.001*
Dyslipidemia	76 (49)	13 (28.9)	63 (57.3)	-	0.001*
Hyperglycemia	16 (10.3)	-	16 (14.5)		-
Carotid duplex sonography (%)					
Carotid stenosis	73 (47)	12 (26.7)	61 (55.5)	-	0.001*
Atheromatous plaque	43 (28)	-	43 (39)	-	
Neurologic manifestations (%)					
History of uremic encephalopathy	32 (20.6)	-	32 (29)	-	-
Uremic seizures (%)					
Generalized tonic-clonic seizures	15 (9.7)	-	15 (13.6)	-	-
Focal seizures	21 (13.5)	-	21 (19)	-	-
Peripheral neuropathy	42 (27.1)	14 (31)	28 (25.5)	-	0.463
Muscle cramps	86 (55.5)	16 (35.6)	70 (63.6)	-	0.001*
Asterixis	110 (71)	-	110 (100)	-	-
Myoclonic jerking	94 (60.6)	-	94 (85)	-	-
Restless leg syndrome	80 (51.6)	-	80 (72.7)	-	-
Focal neurologic manifestations	24 (15.5)	-	24 (21.8)	-	-
Parkinsonism	6 (3.9)	-	6 (5.5)	-	-
Choreo-dystonia	2 (1.3)	-	2 (1.8)	-	-
Dysarthria	3 (1.9)	-	3 (2.7)	-	-
Hemihypoesthesia	9 (5.8)	-	9 (8.2)	-	-
Visual field defects	4 (2.6)	-	4 (3.6)	-	-

Notes: Data are expressed as *n* (%), unless otherwise indicated. [a]Within group comparisons; [b]Between group comparisons (CKD vs. ESKD); *indicates statistically significant values ($p<0.05$).
Abbreviations: CKD: Chronic kidney disease; ESKD: End-stage kidney disease; SD: Standard deviation.

treatment included insulin (n = 27, 64.3%) and oral anti-hyperglycemic agents (n = 42). The oral medications used in different combinations were as follows: sulfonylureas (glimepiride) in 14 patients (33.3%), biguanides (metformin) in 28 patients (66.7%), dipeptidyl peptidase 4 inhibitors (vildagliptin) in 16 patients (38%), and sodium-glucose cotransporter-2 inhibitors (dapagliflozin) in four patients (9.5%). Of the 42 patients with diabetes, 26 (62%) were well-controlled for at least 6 months before the study, while 16 (38%) were poorly controlled on medications.

Some patients with hypertension and/or diabetes (n = 35, 22.6%) were prescribed antiplatelet medications. These medications included aspirin (cyclooxygenase inhibitor; n = 28, 22.6%) and/or clopidogrel (adenosine diphosphate receptor inhibitors; n = 14, 9%).

Atrial fibrillation (AF) was reported in 18% of patients with ESKD, which equates to 28 individuals or 25% of the ESKD patient population. Six patients had been diagnosed with AF before starting hemodialysis, while the rest were diagnosed during the study period. All patients were treated with bisoprolol, a selective beta-blocker, but none were prescribed oral anticoagulants.

3.2. Laboratory investigations

Table 2 presents the results of laboratory investigations for the studied groups. Patients showed significantly higher levels of BUN (p=0.0001), creatinine (p=0.0001), HbA1c (p=0.001), uric acid (p=0.0001), total cholesterol (p=0.0001), low-density lipoprotein (LDL)-C (p=0.001), triglycerides (p=0.0001), and phosphate (p=0.001) compared to controls. Conversely, patients had lower levels of calcium (p=0.01), albumin (p=0.05), hemoglobin (p=0.003), and red blood cell count (p=0.02). The frequency of laboratory abnormalities in patients, ranked from highest to lowest (Table 1), was as follows: anemia (60%, n = 93), hypocalcemia (55.5%, n = 86), hyperuricemia (52%, n = 80), hypertriglyceridemia (49%, n = 76), proteinuria (35.5%, n = 55), hypercholesterolemia (34.2%, n = 53), and hypomagnesemia (29%, n = 45).

Table 2. Laboratory results of patients with CKD, ESKD, and control subjects

Laboratory characteristics	All patients (*n*=155)	CKD (*n*=45)	ESKD (*n*=110)	Control subjects (*n*=30)	*p*-value[a]	*p*-value[b]
Sodium level, mmol/L	120.00–147.23 (134.03±3.67)	132.00–145.00 (136.00±2.84)	120.00–147.23 (132.63±3.04)	132.00–183.23 (138.46±2.48)	0.564	0.428
Potassium level, mmol/L	2.00–5.30 (3.03±0.82)	2.50–5.00 (4.32±0.45)	2.00–5.30 (4.52±0.58)	3.50–5.00 (4.20±0.35)	0.363	0.468
Magnesium level, mg/dL	1.06–2.80 (2.30±0.63)	1.20–2.70 (2.32±0.64)	1.06–2.80 (2.36±0.86)	1.50–2.83 (2.00±0.52)	0.368	0.503
Calcium level, mg/dL	5.30–9.80 (6.36±0.58)	7.00–10.00 (7.57±0.55)	5.30–9.80 (7.08±0.33)	8.40–11.00 (9.39±0.46)	0.010*	0.233
Phosphate level, mg/dL	2.73–9.60 (5.68±1.40)	2.80–8.50 (5.68±1.46)	2.73–9.30 (6.35±1.68)	2.50–4.80 (3.25±0.60)	0.001*	0.708
[c]Blood urea nitrogen, mmol/L	6.00–32.00 (14.32±3.45)	7.00–13.00 (10.85±1.82)	6.00–32.00 (18.56±2.46)	2.80–5.80 (3.46±2.46)	0.0001*	0.010*
[c]Creatinine, µmol/L	185.00–698.00 (356.68±65.38)	185.00–340.00 (253.20±67.23)	185.00–1,480.00 (586.31±83.70)	66.00–121.00 (89.27±8.09)	0.0001*	0.0001*
International normalized ratio, %	1.00–1.50 (1.27±0.05)	1.00–1.50 1.37±0.60)	1.00–1.63 (1.37±0.17)	0.8–1.30 (1.01±0.05)	0.688	1.00
AST, µ/L	13.00–75.00 (30.50±5.46)	20.00–60.00 (25.86±6.46)	13.00–75.00 (45.54±4.56)	15.00–45.00 (25.50±4.52)	0.542	0.353
ALT, µ/L	20.00–106.00 (35.50±5.63)	25.00–58.00 (28.42±6.87)	20.00–106.00 (48.36±5.60)	19.00–56.00 (30.60±4.68)	0.458	0.468
Total bilirubin, µmol/L	3.00–18.00 (10.32±3.38)	3.00–15.00 (9.24±3.60)	4.00–18.00 (14.35±3.56)	3.50–15.00 (9.85±3.36)	0.658	0.546
Albumin, g/L	1.53–4.80 (3.50±0.62)	2.80–4.80 (3.24±0.82)	1.30–4.10 (2.32±0.45)	3.00–5.00 (3.90±0.64)	0.050*	0.030*
Red blood cell count, million/mcL	1.80–5.32 (3.45±0.62)	1.80–5.32 (3.25±0.45)	2.26–4.68 (3.15±0.55)	4.23–6.20 (4.60±0.73)	0.020*	0.658
Hemoglobin, mg/dL	6.00–10.00 (7.42±1.45)	6.20–10.00 (8.50±1.33)	6.00–10.00 (7.86±1.33)	10.80–13.20 (12.20±1.46)	0.003*	0.524
Platelet count,/µL	250.00–375.00 (320.74±35.65)	250.00–375.00 (380.600±40.68)	250.00–350.00 (256.95±50.60)	250.00–450.00 (350.56±30.50)	0.543	0.473
Hemoglobin A1c, %	3.40–8.00 (6.85±1.35)	5.50–8.00 (7.35±2.02)	3.40–8.00 (7.80±1.56)	4.00–5.80 (4.80±0.80)	0.001*	0.144
Uric acid, mg/dL	3.50–12.00 (10.58±1.26)	3.50–10.00 (8.46±1.33)	6.00–12.00 (11.58±2.05)	3.50–6.00 (4.32±1.05)	0.0001*	0.01*
Lipid profile, mg/dL						
Total cholesterol	100–550 (370.50±50.30)	100–300 (250.70±30.60)	100–550 (480.50±70.20)	100–200 (120.60±10.05)	0.0001*	0.0001*
LDL-C	150–270 (200.30±70.40)	150–200 (180.60±50.00)	150–270 (220.50±45.60)	80–150 100.40±8.60)	0.001*	0.248
HDL-C	30–60 (45.20±10.20)	30–60 (40.20±10.50)	30–45 (35.50±10.45)	40–60 (50.60±5.30)	0.01*	0.02
Triglycerides	200–800 (450.56±100.55)	200–400 (330.35±45.32)	200–800 (670.30±85.68)	100–230 (150.46±15.43)	0.0001*	0.0001*
Arterial blood gases						
pH	7.30–7.43 (7.35±0.03)	7.30–7.44 (7.35±0.20)	7.30–7.44 (7.35±0.03)	7.35–7.45 (7.38±0.02)	0.534	0.640
PO_2, mmHg	80.00–99.00 (92.30±3.60)	85.00–99.00 (90.65±4.22)	80.00–99.00 (90.53±3.65)	88.00–100.00 (95.00±3.65)	0.468	0.563
PCO_2, mmHg	23.00–42.00 (32.56±3.80)	27.00–39.00 (35.35±3.70)	23.00–42.00 (36.45±3.80)	32.00–42.00 (38.27±2.65)	0.286	0.332
HCO, MEq/L	20.00–24.00 (18.56±2.03)	20.00–26.00 (23.26±1.72)	20.00–24.00 (16.20±2.34)	22.00–26.00 (22.53±1.81)	0.020*	0.001*
Oxygen saturation, %	80–100 (90.30±2.50)	90–100 (95.80±3.60)	80–100 (90.55±2.30)	90–100 (95.63±2.80)	0.568	0.680

Notes: Data are expressed as range and mean±standard deviation, unless otherwise indicated. [a]Comparison between patients and controls; [b]Comparison between CKD and ESKD; [c]Blood urea nitrogen and creatinine levels represent median levels over the preceding 6 months. *indicates statistically significant values ($p<0.05$).

Abbreviations: ALT: Alanine transaminase; AST: Aspartate transaminase; CKD: Chronic kidney disease; ESKD: End-stage kidney disease; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol.

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Compared with patients with CKD who were not on dialysis, those with ESKD receiving hemodialysis exhibited higher levels of BUN (p=0.0001), creatinine (p=0.0001), uric acid (p=0.01), total cholesterol (p=0.0001), and triglycerides (p=0.0001), and lower levels of high-density lipoprotein (HDL)-C (p=0.02) and bicarbonate (p=0.02). Patients with ESKD also had higher frequencies of hypocalcemia (p=0.001), hypomagnesemia (p=0.001), anemia (p=0.001), proteinuria (p=0.05), hyperuricemia (p=0.001), hypercholesterolemia (p=0.001), and hypertriglyceridemia (p=0.001). Hyperphosphatemia, hypoalbuminemia, and hyperglycemia were only observed in patients with ESKD.

Upon reviewing the medical treatment at presentation, it was found that only 22 (14.2%) patients with hyperlipidemia received statin therapy, specifically atorvastatin, fluvastatin, lovastatin, rosuvastatin, or simvastatin, which are lipid-lowering drugs in the class of hydroxymethylglutaryl-CoA reductase inhibitors.

3.3. Intima-media thickness of the carotid arteries

The median cIMT value in healthy subjects was 1.2 mm, with the 25th, 50th, and 75th percentiles at 0.88 mm, 1.2 mm, and 1.8 mm, respectively. An abnormal cIMT was defined as >1.8 mm. Thickened cIMT, indicative of carotid atherosclerosis, was observed in 47% (n = 73) of the subjects. Atheroma plaques were present in 39% (n = 38) of the subjects (Tables 1 and 3).

3.4. MRI findings

Table 4 and Figure 1 present the MRI findings of the patients under study. The results indicate brain infarctions were the most common finding (78.7%), followed by WMHs (63.2%). Microbleeds were observed in a small number of patients with ESKD (11.6%, n = 18). Bilateral calcification of the basal ganglia was reported in 15.5% (n = 24) of cases. WMHs were characterized by small periventricular and subcortical hyperintense lesions in T2WI and FLAIR, and hypointense in T1WI views of MRI. Microbleeds were identified as focal areas of signal loss in brain parenchyma measuring ≤10 mm on susceptibility-weighted MRI. Lacunar brain infarctions were more prevalent in patients (60.6%, n = 94) than small brain infarctions (15.5%, n = 24; p=0.0001). Bilateral infarctions (45.2%, n = 70) were more common than unilateral ones (33.6%, n = 52; p=0.01). Brain infarctions were located in either the cerebral hemispheres (55.5%, n = 86) or the basal ganglia (23.2%, n = 36). In 58% of patients (n = 90), brain infarctions appeared hypointense in T1WI and apparent diffusion coefficient and hyperintense in DWI, T2WI, and FLAIR, indicating acute/subacute ischemic foci. Encephalomalacia was observed in 32 patients (20.6%) (iso-signal to cerebrospinal fluid; hypointense in DWI and hyperintense in T2WI and FLAIR), suggesting old infarctions. In the absence of clinical stroke symptoms, these lesions may be attributed to silent brain infarcts.

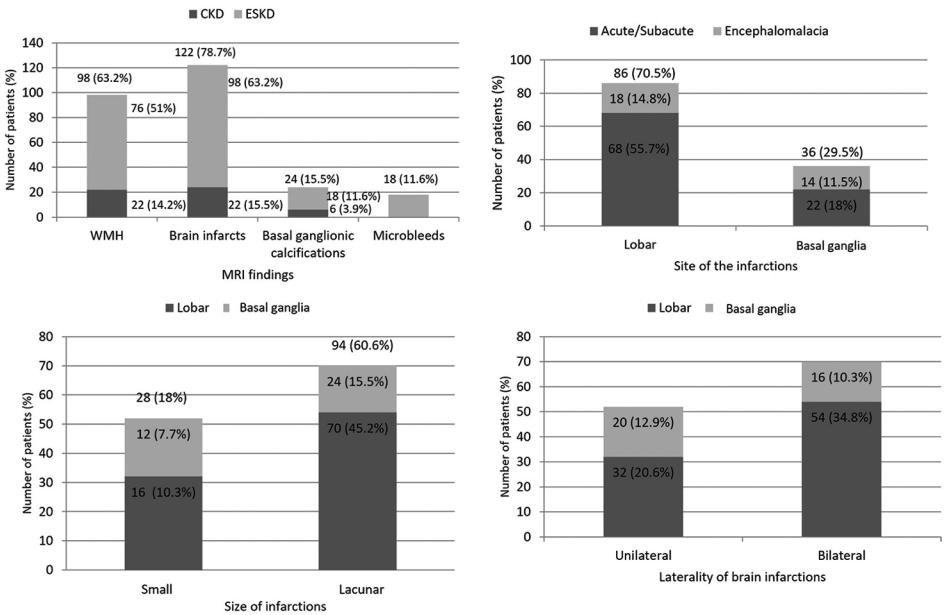


Figure 1. Neuroimaging findings in patients with CKD and ESKD

Abbreviations: CKD: Chronic kidney disease; ESKD: End-stage kidney disease; MRI: Magnetic resonance imaging; WMHs: White matter hyperintensities.

Table 3. Demographic, clinical, laboratory, and neuroimaging characteristics of patients with and without ischemic brain infarctions

Characteristics	All patients (*n*=155) (%)	With brain infarcts (*n*=122, 78.7%)	Without brain infarcts (*n*=33, 21.3%)	*p*-value
Male	70 (45.2)	42 (34.4)	28 (84.8)	0.001*
Female	85 (54.8)	80 (65.6)	5 (15.2)	0.001*
Age, years (range [mean±SD])	45–65 (55.36±4.28)	52–65 (60.52±2.30)	45–65 (52.46±3.60)	0.01*
Duration of uremia, years (range [mean±SD])	3–10 (6.40±1.36)	3–10 (8.23±1.50)	3–7 (5.46±1.82)	0.001*
Causes and comorbid medical conditions				
Hypertension without or with ischemic heart disease	40 (25.8)	30 (24.6)	10 (30.3)	0.126
Diabetes mellitus	16 (10.3)	12 (9.8)	4 (12)	0.543
Hypertension and diabetes	26 (16.8)	20 (16.4)	6 (18.2)	0.01*
Nephritis	73 (47)	60 (49.2)	13 (39.4)	0.236
Atrial fibrillations	24 (15.5)	16 (13)	8 (24.2)	0.126
History of uremic encephalopathy	32 (20.6)	32 (26.2)	0	-
Type of seizures				
Generalized tonic-clonic seizures	15 (9.7)	15 (12.3)	0	-
Focal seizures	21 (13.5)	21 (17.2)	0	-
Focal neurologic manifestations	24 (15.5)	24 (19.7)		
Parkinsonism	6 (3.9)	6 (4.9)	-	-
Left putamen and globus pallidus externa	5 (3.2)	5 (4.1)	-	-
Left caudate and lentiform nuclei	1 (0.7)	1 (0.8)	-	-
Choreo-dystonia	2 (1.3)	2 (1.6)	-	-
Right caudate nucleus	2 (1.3)	2 (1.6)	-	-
Dysarthria	3 (1.9)	3 (2.5)	-	-
Right insular infarction	3 (1.9)	3 (3.5)	-	-
Hemihyposthesia	9 (5.8)	9 (7.4)	-	-
Parietal lobe infarction	9 (5.8)	9 (7.4)	-	-
Visual field defects (occipital lobe infarction)	4 (2.6)	4 (3.3)	-	-
Hyponatremia	22 (14)	22 (18)	0	-
Hypokalemia	30 (19.4)	30 (24.6)	0	-
Hypocalcemia	86 (55.5)	78 (63.9)	8 (24.2)	0.0001*
Hypomagnesaemia	45 (29)	45 (36.9)	-	-
Hyperphosphatemia	52 (33.5)	42 (34.4)	6 (18.2)	0.01*
Anemia	93 (60)	81 (66.4)	12 (36.4)	0.0001*
Hypoalbuminemia	96 (62)	88 (72)	8 (24.2)	0.0001*
Proteinuria	55 (35.5)	49 (40.2)	6 (18.2)	0.0001*
Hyperparathyroidism	36 (23.2)	36 (29.5)	0	-
Hyperuricemia	56 (36)	51 (41.8)	5 (15.2)	0.0001*
Hypercholesterolemia	53 (34.2)	48 (39.4)	5 (15.2)	0.001*
Dyslipidemia	76 (49)	65 (53.3)	11 (33.3)	0.001*
Hyperglycemia	16 (10.3)	16 (14.5)	-	-
Carotid stenosis	73 (47)	58 (45.9)	15 (45.5)	0.546
Atheromatous plaque	43 (28)	32 (26.2)	11 (33.3)	0.328
WMHs	98 (63.2)	76 (62.3)	22 (66.7)	0.658

Notes: Data are expressed as *n* (%), unless otherwise indicated.*indicates statistically significant values ($p<0.05$).
Abbreviations: CKD: Chronic kidney disease; ESKD: End-stage kidney disease; SD: Standard deviation; WMHs: White matter hyperintensities.

Table 4. Neuroimaging findings in patients with CKD and ESKD

Neuroimaging findings	All patients (*n*=155) (%)	CKD (*n*=45) (%)	ESKD (*n*=110) (%)	*p*-value
WMHs	98 (63.2)	22 (14.2)	76 (49)	0.001*
Ischemic brain infarctions	122 (78.7)	24 (15.5)	98 (63.2)	0.0001*
Cerebral brain infarctions (lobar)	86 (70.5)	18 (11.6)	68 (43.9)	0.001*
Onset				
Acute/subacute infarctions	68 (55.5)	-	68 (43.9)	
Encephalomalacia	18 (11.6)	-	18 (11.6)	
Size				
Small infarctions	16 (10.3)	-	16 (10.3)	
Lacunar infarctions	70 (45.2)	18 (11.6)	52 (33.5)	<0.05*
Laterality				
Unilateral	32 (20.6)	5 (3.2)	27 (17.4)	
Bilateral	54 (34.8)	13 (8.4)	39 (25.2)	
Basal ganglionic ischemic infarctions	36 (23.2)	6 (3.9)	30 (19.4)	0.01*
Onset				
Acute/subacute infarctions	22 (14.2)	-	22 (14.2)	
Encephalomalacia	14 (9)	-	14 (9)	
Size				
Small infarctions	12 (7.7)	-	12 (7.7)	
Lacunar infarctions	24 (15.5)	6 (3.9)	18 (11.6)	<0.05*
Laterality				
Unilateral	20 (12.9)		20 (12.9)	
Bilateral	16 (10.3)	6 (3.9)	10 (6.5)	
Bilateral basal ganglionic calcification (lentiform nuclei)	24 (15.5)	6 (3.9)	18 (11.6)	<0.05*
Microbleeds	18 (11.6)		18 (11.6)	

Notes: Data are expressed as *n* (%), unless otherwise indicated. *$p<0.05$ is considered statistically significant. *P* values indicate comparisons between patients on dialysis and patients not on hemodialysis.
Abbreviations: CKD: Chronic kidney disease; ESKD: End-stage kidney disease; WMHs: White matter hyperintensities.

3.5. Clinical manifestations of the studied group

3.5.1. Uremic symptoms

Patients with kidney disease reported symptoms such as tiredness, itching, loss of appetite, nausea, vomiting, breathlessness, ankle edema, and reduced urine output.

3.5.2. Neurological manifestations

Neurological symptoms reported in patients included asterixis (negative myoclonus; 71%), myoclonic jerking (positive myoclonus; 60.6%, *n* = 94), muscle cramps (55.5%, *n* = 86), restless leg syndrome (RLS; 51.6%, *n* = 80), uremic seizures (23.2%, *n* = 36), peripheral neuropathy (27%, *n* = 42), and uremic encephalopathy (20.6%, *n* = 32). Asterixis, myoclonic jerks, RLS, seizures, and encephalopathy were only observed in patients with ESKD. Muscle cramps were common in patients with hypocalcemia, while myoclonic jerks were frequent in patients with uremic encephalopathy. Myoclonic jerking was characterized by brief, irregular, involuntary movements involving various body parts. RLS was described as unpleasant leg sensations with an urge to move the leg, often accompanied by sensory disturbances and periodic leg movements. Peripheral neuropathy, seen in diabetic patients, presented as numbness, burning sensation, and hypoesthesia. It manifested as a stocking distribution of numbness, burning sensation, and hypoesthesia on pin-prick examination. Uremic seizures were either generalized tonic-clonic seizures (*n* =15, 9.7%) or focal seizures (sensorimotor; *n* = 21, 13.5%). Patients with seizures had hypocalcemia, a history of uremic encephalopathy (44.4%; *n* = 16), bilateral basal ganglia calcifications (33.3%; *n* =12), WMHs (100%, *n* = 36), and lacunar brain infarctions (100%, *n* =36). None of the seizure patients were taking antiepileptic medications during the study period.

During the study period, 19.7% (*n* = 24) of patients among 122 with ischemic brain infarctions developed cerebrovascular strokes. The manifestations of stroke

varied depending on the location of the brain infarction (Table 1). Dysarthria and somatosensory deficits were observed in 9.8% of patients (n = 12) with small cerebral ischemic infarctions in the insula and parietal/parieto-temporal brain regions, respectively. Visual field defects and homonymous hemianopia were reported in 2.6% of patients (n = 4) with ischemic infarction in the occipital lobes (Table 1 and Figure 2A). The patients with neurologic deficits were aged between 55 and 65 years and experienced strokes 12–24 months after the onset of uremia. They had chronic hypertension and diabetes. Extrapyramidal movement disorders were observed in 5.2% of patients (n = 8) with ischemic infarctions in the basal ganglia, with six exhibiting parkinsonism and two showing choreo-dystonia. Patients with parkinsonism, characterized by unilateral rest tremors, were aged between 55 and 60 years and developed symptoms 10–24 months after the onset of uremia. They also had diabetic nephropathy. Lesions were found in the putamen, globus pallidus externa, caudate, and lentiform nuclei (Figure 2B). Patients with choreo-dystonic involuntary movements (unilateral) were aged 58 and 62 years and developed symptoms 12–16 months after the onset of uremia, both with a history of chronic hypertension and diabetes. Chorea was characterized by abrupt, irregular, arrhythmic, continuous, and rapid involuntary movements, while dystonia involved sustained, patterned, and repetitive muscle contractions leading to twisting movements or abnormal postures, with lesions in the caudate nucleus. The laboratory profiles of patients with cerebrovascular stroke did not significantly differ from those of other patients with ESKD. All patients with cerebrovascular stroke exhibited WMHs and bilateral lacunar ischemic infarctions.

3.6. Comparative statistical analyses of demographic, clinical, and laboratory variables between patients with and without brain infarctions

Patients with ESKD had a higher incidence of cerebral (p=0.001) and basal ganglionic (p=0.01) infarctions compared to those not on dialysis. Brain infarctions were more common in females (p=0.0001), older individuals (p=0.0001), longer duration of kidney disease (p=0.001), those with both hypertension and diabetes (p=0.0001), hypocalcemia (p=0.0001), hyperphosphatemia (p=0.01), anemia (p=0.0001), hypoalbuminemia (p=0.01), hypercholesterolemia (p=0.001), dyslipidemia (p=0.001), and hyperuricemia (p=0.0001) (Table 3). No specific laboratory abnormalities or MRI findings distinguished patients with myoclonic jerks or RLS from other patients

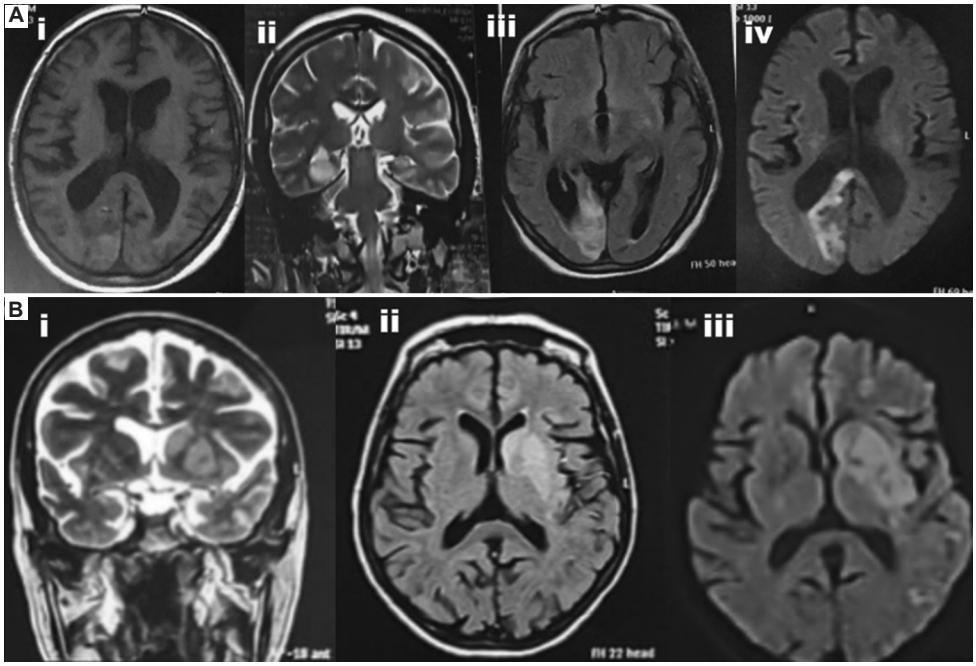


Figure 2. Magnetic resonance imaging (MRI) of patients with uremia and cerebrovascular stroke. (A) MRI-brain axial and coronal views) of a 62-year-old man. He had hypertension for 10 years and uremia at the age of 55 years. He developed a visual field defect. He had a lesion in the right occipital lobe. The lesion is hypointense in (i) T1WI and hyperintense in (ii) TWI, (iii) FLAIR, and (iv) DWI (i.e., ischemic lesion). (B) MRI-brain (coronal and axial views) of a 60-year-old male (age at presentation). He had diabetes for 8 years, developed uremia at the age of 55 years, and is on regular hemodialysis. He developed right-sided rest tremors and rigidity (parkinsonism) during the period of the study. He had MRI lesions in the left caudate and lentiform nuclei. Lesions are hypointense in TWI and hyperintense in (i) T2WI, (ii) FLAIR, and (iii) DWI (i.e., ischemic lesions).
Abbreviations: DWI: Diffusion-weighted imaging; FLAIR: Fluid-attenuated inversion recovery; T1WI: T1-weighted imaging; T2WI: T2-weighted imaging.

with ESKD. In addition, there were no significant differences in the prevalence of abnormal cIMT between those with and without ischemic brain infarctions.

3.7. Correlation analyses

The correlation analysis between variables showed significant correlations between hypertension and hyperglycemia, hypercholesterolemia (p=0.001), hypertriglyceridemia (p=0.001), hyperuricemia (p=0.001), and cIMT (p=0.001). Hyperuricemia was significantly correlated with hypercholesterolemia (p=0.001), hypertriglyceridemia (p=0.001), and cIMT (p=0.001). Hypertriglyceridemia significantly correlated with hypercholesterolemia (p=0.001) and cIMT (p=0.001). Hypoalbuminemia and proteinuria were significantly correlated (p=0.001).

3.8. Multiple regression analysis

A multiple regression analysis revealed that the presence of brain infarcts, compared to those without brain infarctions, was independently associated with the severity of kidney disease (OR = 6.32, 95% CI = 3.20–15.45, p=0.0001) after adjusting for age and sex, hypertension (OR = 8.34, 95% CI = 5.46–16.25, p=0.0001), hypertriglyceridemia (OR = 5.20, 95% CI = 2.45–12.33, p=0.001), hyperuricemia (OR = 3.40, 95% CI = 1.40–6.32, p=0.001), anemia (OR = 2.43, 95% CI = 1.60–5.28, p=0.01), hypoalbuminemia (OR = 3.25, 95% CI = 1.64–8.42, p=0.01), and albuminuria (OR = 2.53, 95% CI = 1.20–5.42, p=0.03). Notably, the presence of diabetes, hypercholesterolemia, hyperphosphatemia, thickened cIMT, WMHs, type of antihypertensive drugs, oral hypoglycemic medications, and the use of statins or antiplatelet medications did not show significant odds ratios (95% CI) in the presence of ischemic brain infarctions.

3.9. Management of patients with silent brain infarctions and cerebrovascular stroke

The treatment approach included optimizing renal replacement therapy, correcting associated metabolic derangements, using antiplatelets (aspirin and/or clopidogrel), and addressing comorbid medical conditions (such as hyperlipidemia, hyperuricemia, anemia, hypocalcemia, and hyperglycemia). Statins were prescribed for hyperlipidemia and allopurinol for hyperuricemia. Oral anticoagulants were not part of the management. Supportive or symptomatic treatments were also provided, such as L-DOPA/carbidopa for parkinsonism, tetrabenazine for chorea, dopamine agonists and gabapentin for RLS, and carbamazepine for seizures.

3.10. Clinical outcome of patients with cerebrovascular stroke

A follow-up of patients who developed a stroke was conducted for over 6 months, which showed persistent neurological manifestations. Antiplatelet and statins were continued to prevent recurrent ischemic strokes.

4. Discussion

This cohort study was conducted on a group of patients with uremia due to CKD over 2 years to estimate the prevalence of silent brain infarctions and the development of cerebrovascular stroke in these patients, along with the associated vascular and non-vascular risk factors and the manifestations and clinical outcomes of cerebrovascular strokes.

In this study, silent brain infarctions were the most common findings in CKD patients, with lacunar (60.7%) and small (18%) brain infarctions being predominant, indicating occlusion of small brain vessels. Ischemic brain infarctions were more frequent in the cerebral hemisphere compared to the basal ganglia (70.5% versus 14.2% or 5:1). Studies have shown a link between reduced eGFR due to CKD and subclinical cerebral brain infarctions and small vessel disease.[11,18,23-26] However, most studies reported lower frequencies (around 50%) of ischemic brain infarctions in patients with CKD. This could be explained by the fact that approximately 70% of the included patients in this study had ESKD. In this study, WMHs were present in 63.2% of patients, with no significant difference in frequency between patients with and without ischemic infarctions.[27] WMHs were not independently associated with silent brain infarctions. They are non-specific findings and can be present in various conditions as hypertension, diabetes, multiple sclerosis, and pyridoxine deficiency.[28,29] WMHs have also been known as leukoaraiosis. The pathogenesis of WMHs is different from silent ischemic brain infarctions. They are characterized by neuronal loss, demyelination, and gliosis.[28] While lacunar ischemic brain infarctions represent a neuropathological change involving hyalinosis of the small end-perforating arterioles in the white matter of the brain.[30] Therefore, many researchers considered ischemic brain infarctions and not WMHs as the better marker for lacunar stroke in brain MRI images. In contrast, some studies have linked CKD with WMHs, albuminuria, and decreased eGFR.[31] This study found that ESKD was linked to ischemic brain infarctions regardless of other risk factors. Previous studies have shown that kidney dysfunction at various stages is independently associated with a higher risk of cerebrovascular disease, separate from traditional risk factors.[32]

In this study, hypertension was found in 42.6% of patients with CKD, of whom 41% had brain infarctions. Previous studies have reported hypertension in 67–92% of patients with CKD.[6] It is unclear in this study if hypertensive nephrosclerosis was the cause of uremia. Hypertension

commonly affects the small perforating arteries in various regions of the body, including the kidney and brain,[33] causing lipohyalinosis of subcortical penetrating arteries and the accumulation of proteins in the arteriolar subendothelial space (hyalinosis), which often extends into the media.[12,17] This is known as arteriosclerosis, a characteristic feature of lacunar brain stroke. Hypertensive nephrosclerosis is characterized by subintimal hyalinosis, medial vascular smooth muscle cells replaced by connective tissue, and glomerular and tubular atrophy with interstitial fibrosis.[34] In addition, hypertension accelerates the progression of systemic and cerebral atherosclerosis.[35] In this study, patients were well-controlled on different groups of antihypertensive medications, including ACE inhibitors (63.6%), potassium-sparing diuretics (15.2%), and beta blockers and calcium channel blockers (21%). There were no significant differences in the frequency of ischemic brain infarctions based on the type of antihypertensive medication used.

In this study, diabetes was found in 27% of patients, among whom 26% were diagnosed with diabetic nephropathy before developing uremia. This same group of patients also had brain infarctions. Previous studies have shown a significant association between CKD, silent brain infarctions, cardiovascular disease, metabolic syndrome, and diabetes.[7] The pathological features of diabetic CKD include glomerular hypertrophy, thickened glomerular basement membrane, and vascular hyalinosis.[36] It is also known that insulin resistance plays a crucial role in developing and progressing systemic and cerebral atherosclerosis and arteriosclerosis. Diabetes is often accompanied by hyperlipidemia,[37] a risk factor for atherosclerosis. The majority of patients had been on insulin and oral hypoglycemic medications (e.g., sulfonylureas, biguanides [metformin], dipeptidyl peptidase 4 inhibitors, and sodium-glucose cotransporter-2 inhibitors) for at least 6 months before being included in the study, while 38% of them had poorly controlled diabetes.

In addition, in this study, 73 patients (47%) with both hypertension and diabetes had carotid stenosis, and 28% of them had atheroma. Carotid atherosclerosis was not found to be independently associated with ischemic brain infarctions, but its potential contribution to ischemic brain infarctions and cerebrovascular stroke in patients with uremia cannot be ruled out. Studies have shown an increase in cIMT and increased stiffness of carotid arteries in patients with CKD.[15] In addition, there is a higher risk of frequent rupture of atheromatous lesions in patients with CKD, independent of traditional cardiovascular risk factors.[38]

In this study, a small percentage of participants had AF (18%). However, AF was not a predictor of ischemic brain infarctions. There was also no observed association between AF and hypertension. It remains unclear if ESKD was the underlying cause of AF in this patient group. Previous research has shown that the prevalence of AF in ESKD patients was 11.6%, with an overall incidence of 2.7/100 patient-years.[5] Patients with AF were prescribed bisoprolol, a selective beta-blocker, but none were given oral anticoagulants to prevent thrombotic/embolic events associated with AF.

Over half of the patients with ischemic brain infarctions had hypertriglyceridemia (dyslipidemia) with or without hypercholesterolemia. Hypertriglyceridemia was significantly linked to hypertension, hyperglycemia, hyperuricemia, hypercholesterolemia, and low HDL cholesterol levels. In addition, hyperlipidemia was independently linked to ischemic brain infarctions regardless of other risk factors. Studies indicated that excess cholesterol in the blood can lead to plaque formation, which can narrow or block blood vessels, causing ruptures and thromboembolic brain infarctions.[37]

It has been noted that dyslipidemia in patients with CKD, especially ESKD, may be atherogenic or uremic. Atherogenic dyslipidemia, common in insulin-resistant patients, is characterized by high total cholesterol, LDL cholesterol, and triglyceride levels, and low HDL cholesterol levels. Uremic dyslipidemia, on the other hand, is characterized by nearly normal LDL and HDL cholesterol levels but high triglyceride levels.[8] Studies indicated that progressive proteinuria in CKD can disrupt lipoprotein transport, leading to elevated triglycerides, very LDL, and LDL cholesterol levels, and reduced HDL cholesterol levels. Furthermore, progressive proteinuria in CKD can upregulate hydroxymethylglutaryl-CoA reductase, causing hypercholesterolemia and increased LDL synthesis. Uremia can also alter the structure and function of HDL, and the loss of lecithin-cholesterol acyltransferase in urine can result in low HDL levels and impaired maturation of HDL-3 to cholesterol-rich HDL-2.[38,39] Only 14.2% of patients with hyperlipidemia in the study received statin treatment, indicating that hyperlipidemia/dyslipidemia was often overlooked and undertreated in kidney disease patients. We also found that statins were not associated with a reduced risk of ischemic brain infarctions.

In this study, approximately 42% of patients with ischemic brain infarctions were found to have hyperuricemia. Hyperuricemia was significantly associated with hypertension, hyperglycemia, and hyperlipidemia. In addition, hyperuricemia was independently linked to ischemic brain infarctions regardless of other risk factors. It is known that uric acid is a major antioxidant in human blood, with two-thirds normally excreted in the

urine.[40-42] CKD impairs uric acid excretion, especially in the presence of insulin resistance and increased renal vascular resistance. In the atherosclerotic environment of CKD, uric acid's antioxidant properties can paradoxically become prooxidant (an antioxidant–prooxidant urate redox shuttle),[41,42] leading to increased reactive oxygen species, arterial endothelial damage, oxidation of lipoproteins in atherosclerotic plaques, and the development of renal fibrosis and glomerulosclerosis. During the study period, hyperuricemia was diagnosed in all patients, none of whom had received treatment for the condition before being included. This suggests that hyperuricemia may have been overlooked in patients with CKD.

Anemia was present in 60% of patients, while ischemic brain infarctions were found in 66% of patients with ESKD. Anemia was identified as an independent variable associated with the presence of ischemic brain infarction. Studies have shown that CKD and anemia elevate the risk of stroke, with individuals without anemia showing a slight, non-significant increase in stroke risk.[43] It is important to note that the correction of anemia in CKD patients should adhere to the Kidney Disease Outcomes Quality Initiative guidelines, aiming to raise hemoglobin levels to between 11 and 12 g/dL. Large randomized clinical trials have demonstrated that treating anemia with erythropoietin-stimulating agents in hemodialysis patients can heighten the risk of stroke.[44]

In this study, the prevalence of hypoalbuminemia was significantly higher in patients with ischemic brain infarctions (74% vs. 24%). In addition, albuminuria was substantially more common in patients with ischemic brain infarctions (40% vs. 18%, p=0.001). Hypoalbuminemia showed a significant correlation with proteinuria. Both hypoalbuminemia and albuminuria were independently linked to ischemic brain infarctions. Kidney damage often leads to progressive proteinuria, resulting in hypoalbuminemia. Research has shown a higher risk of cerebrovascular stroke in patients with kidney disease and macroalbuminuria compared to those with microalbuminuria.[45] Proteinuria is strongly associated with hypertension and other cardiovascular risk factors.[46] Albuminuria not only indicates localized renal damage but also serves as a biomarker for generalized endothelial dysfunction, increasing the risk of vascular events such as ischemic brain infarctions and stroke. Low serum albumin levels may indicate factors that influence the atherosclerotic process, platelet function, blood viscosity, free fatty acid transport, and antioxidant levels.[47]

In this study, approximately 20% of individuals with ischemic infarctions developed cerebrovascular stroke during the period of the study. Patients with cerebrovascular stroke accounted for approximately 22% of those with ESKD. No specific laboratory or MRI findings distinguished this subgroup of patients from the rest with ESKD. Studies have shown that individuals with ESRD have a significantly higher incidence of stroke, 8–10 times greater than the general population, with rates ranging from 10 to 33/1,000 patients/year.[48] The prevalence of stroke was 17% among dialysis patients compared to 10% among non-dialysis CKD patients and 4% in the general population.[49,50] Previous research has indicated an inverse linear relationship between eGFR and the risk of stroke (ischemic or hemorrhagic), with the risk of stroke increasing by 7% for every 10 mL/min/1.73 m^2 decrease in eGFR[12] and by 10%/25 mg/mmol increase in the albumin-to-creatinine ratio independent of eGFR.[18] Focal neurologic stroke manifestations included dysarthria, hemihypoesthesia, and visual field defects. Eight out of 36 patients with basal ganglionic brain infarctions (22%) experienced an acute onset of extrapyramidal movement disorders, including parkinsonism (n = 6) and chorea-dystonia (n = 2). Reviewing the literature revealed case reports of extrapyramidal movement disorders in uremic patients, including parkinsonism,[10,51-53] chorea,[10,54,55] and dystonia.[10,56] In a previous study,[10] we examined 70 adults (mean age: 45.87 ± 3.36 years) with uremia due to CKD with a duration of uremia of 5.5 ± 1.5 years. Basal ganglia lesions were found in 21.4% of the patients, with 8.6% developing extrapyramidal movement disorders after ~10 months from the onset of uremia. These disorders were attributed to basal ganglia edema and ischemic infarctions.

The results of this study highlight the bidirectional causal relationship between vascular brain diseases and CKD and uremic syndrome. It has been indicated that the uremic environment also significantly contributes to and exacerbates systemic and cerebral vascular damage, arteriosclerosis, atherosclerosis, and calcification. This environment includes uremic toxins, anemia, chronic inflammation, endothelial dysfunction, cerebral hypoperfusion, impaired autoregulation, increased cytokine release, oxidative stress, reduced nitric oxide availability, hyperhomocysteinemia, dyslipidemia, platelet dysfunction, increased aggregation, and elevated levels of hemostatic factors.[3,4]

In this study, the treatment of acute cerebrovascular stroke in CKD involved several approaches optimizing renal replacement therapy (such as more frequent and adequate dialysis), correcting associated metabolic imbalances, addressing the underlying cause of CKD and its comorbidities, using antiplatelets to prevent recurrent strokes, and providing supportive or symptomatic care (e.g., levodopa therapy for parkinsonism, anti-dopaminergic

drugs for chorea). ACE2 inhibitors or blockers are the preferred treatment for hypertension in patients with CKD. These medications reduce the risk of cardiovascular and cerebrovascular events and slow the progression of CKD.[57,58] Studies have demonstrated the effectiveness of sodium-dependent glucose transporter inhibitors in managing diabetes, diabetic nephropathy, cardiac disease, stroke, and kidney disease.[59] Statins have been proven effective in treating hyperlipidemia in CKD patients.[60] Allopurinol is the primary treatment of hyperuricemia in CKD patients, although febuxostat has emerged as an alternative to allopurinol.[61]

We and others agree on the use of antiplatelets (aspirin or clopidogrel) in the treatment of patients with silent ischemic brain infarction and for preventive purposes. However, the management of silent infarctions remains a topic of debate. It has been recommended that the use of antiplatelets for preventing ischemic stroke in patients with CKD should adhere to the clinical guidelines of the Kidney Disease Outcomes Quality Initiative. There is evidence that supports the effectiveness of antiplatelets in preventing stroke in non-dialysis CKD patients.[62] Furthermore, prior research has suggested that patients with CKD, including those with ESKD, can be treated for acute stroke similarly to the general population,[63] including the use of thrombolysis (e.g., tissue plasminogen activator) and endovascular thrombectomy.[64] However, these studies have also noted a higher risk of intracerebral hemorrhage with tissue plasminogen activator in CKD patients compared to the general population.[65]

We did not prescribe anticoagulants to prevent stroke in patients with AF and CKD due to the controversial nature of their use in this population. Studies have shown that warfarin, a commonly used anticoagulant, may increase the risk of bleeding in CKD patients on dialysis without significantly reducing the risk of stroke compared to no anticoagulation. Warfarin works by affecting vitamin K-dependent clotting factors and anticoagulant proteins.[66] Some studies suggest that apixaban, a newer anticoagulant that selectively inhibits factor Xa, may be a safer and more effective option for these patients. Authors have recommended switching from warfarin to apixaban in this population.[67] It is essential to closely monitor the international normalized ratio when using anticoagulants in patients with ESKD to adjust the dosage and prevent bleeding.

In this study, patients with a history of cerebrovascular stroke more than 6 months prior were followed up, and it was found that they still had persistent neurological symptoms. The clinical poor outcomes observed may be due to the combination of uremia and underlying microvascular diseases such as hypertension and diabetes in predisposed patients. Previous studies have shown different outcomes, with 20% experiencing complete improvement, 50% showing improvement with remaining deficits, and 30% with no improvement.[51,52]

5. Study limitations

The study primarily included patients with ESKD undergoing dialysis, potentially underestimating the frequency and progression of ischemic brain infarctions in early stages of kidney disease. This has been explained by the fact that patients were recruited from tertiary referral hospitals, where severe cases are commonly referred for dialysis and complications management, while ESKD patients are often treated in private clinics. The restrictive inclusion/exclusion criteria (e.g., exclusion of patients with previous ischemic or hemorrhagic strokes, but many CKD patients have mixed pathologies) may limit generalizability. In addition, the study only focused on the clinical outcomes of patients who experienced cerebrovascular stroke due to ischemic brain infarctions during the follow-up period. We did not assess the functional outcome of patients with CKD who developed stroke. Future studies should examine the functional outcomes and quality of life of CKD patients who develop cerebrovascular stroke, considering different neurological complications of CKD, such as cognitive impairment. The temporal relationship between brain infarction development and hemodialysis initiation was not established. Previous reports suggest a higher stroke risk within the first 30 days of starting hemodialysis due to rapid intravascular volume changes. Recurrent seizures were observed in 23% of patients, often coinciding with renal function decline and a history of uremic encephalopathy. While all patients had ischemic brain infarctions, the direct link between infarctions and seizures was not definitive. Uremic toxins and metabolic disturbances are potential contributors to uremic seizures. Antiseizure medications recommended for CKD patients undergoing hemodialysis are those primarily metabolized by the liver (e.g., carbamazepine, phenytoin, and valproate), with potential dose adjustments needed due to dialysis clearance. Monitoring free drug levels is crucial in CKD patients with hypoalbuminemia to prevent drug toxicity.[68,69]

6. Conclusion

There is limited and conflicting data in the literature on the types, prevalence, treatment, and prevention of cerebral infarctions caused by uremic syndrome in CKD due to the diverse range of causes and comorbidities in the a*t*-risk population. This study systematically examined a cohort of patients at various stages of kidney failure to

investigate the occurrence of ischemic brain infarctions and cerebrovascular strokes. The findings revealed a high incidence of silent cerebral infarctions in CKD patients, affecting over two-thirds of individuals, with small bilateral lacunar infarctions being the most common type. Approximately 22% of patients with silent ischemic infarctions developed cerebrovascular strokes. Risk factors for ischemic infarctions in CKD included advanced renal dysfunction, hypertension, diabetes, hyperlipidemia, hyperuricemia, anemia, hypoalbuminemia, and albuminuria. Recommendations were provided for managing these patients and preventing recurrent strokes, emphasizing the importance of addressing all vascular risk factors and implementing preventive measures. The study also highlighted the underdiagnosis and undertreatment of hyperlipidemia and hyperuricemia in this patient population. Patients with cerebrovascular stroke in ESKD often have a poor prognosis due to ongoing neuronal damage from uremic brain injury and underlying microvascular disease.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: All authors
Formal analysis: All authors
Investigation: All authors
Methodology: All authors
Writing–original draft: Sherifa A. Hamed
Writing–review & editing: All authors

Ethics approval and consent to participate

The study protocol was conducted in accordance with the Ethical Standards of the National Research Committee of the Faculty of Medicine of Assiut (ID: AUFM_SH_0135/2021) and Al-Azhar University Hospital, Assiut, Egypt, which is comparable to the ethical standards of the Helsinki Declaration and its later amendments. Informed consent to participate in the study was obtained from patients or their relatives.

Consent for publication

Written informed consent was obtained from all participants to publish their data.

Availability of data

Data will be made available through the corresponding authors upon reasonable request.

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Appendix

Table A1. Strengthening the reporting of observational studies in epidemiology (STROBE) statement

Parameter	Item no	Recommendation	Page #
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract	1
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3
Objectives	3	State-specific objectives, including any pre-specified hypotheses	3
Methods			
Study design	4	Present key elements of the study design early in the paper	4
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	4
Participants	6	(a) Cohort study—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up case–control study—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls cross-sectional study—Give the eligibility criteria, and the sources and methods of the selection of participants	4
		(b) Cohort study—For matched studies, give the matching criteria and the number of exposed and unexposed case–control study—For matched studies, give the matching criteria and the number of controls per case	4
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	4
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe the comparability of assessment methods if there is more than one group	4,5
Bias	9	Describe any efforts to address potential sources of bias	4
Study size	10	Explain how the study size was arrived at	4
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	4,5
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	6
		(b) Describe any methods used to examine subgroups and interactions	6
		(c) Explain how missing data were addressed	6
		(d) Cohort study—If applicable, explain how loss to follow-up was addressed case–control study—If applicable, explain how matching of cases and controls was addressed cross-sectional study—If applicable, describe analytical methods taking account of sampling strategy	6
		(e) Describe any sensitivity analyses	-
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—e.g., numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analyzed	7–11
		(b) Give reasons for non-participation at each stage	-
		(c) Consider the use of a flow diagram	-
Descriptive data	14*	(a) Give characteristics of study participants (e.g., demographic, clinical, social) and information on exposures and potential confounders	7
		(b) Indicate the number of participants with missing data for each variable of interest	-
		(c) Cohort study—Summarize follow-up time (e.g., average and total amount)	-
Outcome data	15*	Cohort study—Report numbers of outcome events or summary measures over time	7–11
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	-
		Cross-sectional study—Report numbers of outcome events or summary measures	-

(Cont'd)

Table A1. ***(Continued)***

Parameter	Item no	Recommendation	Page #
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (e.g., 95% confidence interval). Make clear which confounders were adjusted for and why they were included	7–11
		(b) Report category boundaries when continuous variables were categorized	-
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	-
Other analyses	17	Report other analyses done—e.g., analyses of subgroups and interactions, and sensitivity analyses	-
Discussion			
Key results	18	Summarize key results with reference to study objectives	12
Limitations	19	Discuss limitations of the study, considering sources of potential bias or imprecision. Discuss both the direction and the magnitude of any potential bias	17
Interpretation	20	Give a cautious overall interpretation of results, considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	12–18
Generalizability	21	Discuss the generalizability (external validity) of the study results	17
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	20

Notes: An explanation and elaboration article discusses each checklist item, gives methodological background, and provides published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the websites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org. *Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

ACCSCIENCE PUBLISHING

Advanced Neurology

CASE REPORT

Guillain–Barré syndrome with positive anti-sulfatide antibodies (Paraplegia with bilateral facial nerve palsy): A case report and retrospective analysis

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Abstract

Guillain–Barré syndrome (GBS) is an autoimmune inflammatory neuropathy frequently triggered by infections such as the Epstein–Barr virus and influenza B virus. While cranial nerve involvement occurs in nearly half of the cases, atypical presentations, including paraplegia with bilateral facial nerve palsy, are extremely rare. Herein, we report a 42-year-old female presenting with progressive lumbosacral pain, paraplegia, and bilateral facial nerve palsy. Diagnostic findings included demyelinating changes on electromyography, protein-cell separation in cerebrospinal fluid, and elevated anti-sulfatide immunoglobulin (Ig)G antibodies, confirming acute inflammatory demyelinating polyneuropathy. Treatment with intravenous Ig and adjunct therapies resulted in significant recovery and improved quality of life. This case emphasizes the diagnostic role of anti-sulfatide antibody testing in atypical GBS and highlights the potential of sulfatide-targeted therapies as novel interventions.

Keywords: Guillain–Barré syndrome; Anti-ganglioside antibodies; Case report; Paraplegia; Nerve palsy

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1. Introduction

Guillain–Barré syndrome (GBS) is an immune-mediated disease typically triggered by infections, affecting peripheral nerves and nerve roots. Annually, about 100,000 new cases are reported worldwide, making it a common cause of acute neuromuscular weakness and delayed paralysis.[1] Myelin in the central and peripheral nervous systems is mainly composed of sulfatide. Sulfatide levels influence the normal function of myelinated axons and nerve conduction velocities, playing a crucial role in maintaining the structural and physiological integrity of the nerve sheath membrane.[2] Pathologically, sulfatide promotes the lateral growth of myelin sheaths, regulates their sorting and assembly, and affects the normal function of myelin proteins.[3]

The current standard treatment for GBS includes intravenous immunoglobulin (Ig) and plasmapheresis, which aim to modulate the immune response and halt disease progression. For atypical GBS variants, such as those involving cranial nerve palsy or paraplegia, the same therapeutic options are applied. However, supportive measures

such as nerve growth factor and vitamin supplementation are often integrated into care to promote nerve repair.[4] Early intervention remains critical to improving outcomes, as symptoms often peak within 2–4 weeks.

Anti-sulfatide antibody-positive GBS has gained attention in recent years, but its diverse clinical manifestations and fluctuating course pose significant diagnostic and therapeutic challenges for physicians. Herein, we describe a case of anti-sulfatide antibody-positive GBS with paraplegia and bilateral facial nerve palsy, a rare and atypical form of GBS. Through this case report, we explore the potential of sulfatide therapy, aiming to enable clinicians to diagnose and treat this type of atypical GBS more swiftly and accurately, thereby alleviating patient suffering and expediting recovery.

2. Case presentation

A 42-year-old Chinese woman (Patient ID 4Z0389000297) was admitted with progressive low back and buttock pain, limb numbness, and weakness. 9 days before admission, she developed persistent, unexplained pain in the lower back and buttocks, which was aggravated by activity. The next day, pain, numbness, and weakness appeared in both lower limbs, most pronounced in the dorsal and dorsolateral regions. Acupressure relieved the back pain but exacerbated the numbness and weakness in her legs.

Five days before admission, she experienced numbness of the tongue and sought care at a local hospital, where she received mannitol (150 mg). Her symptoms worsened, and 1 day before admission, she developed left-sided weakness of eyelid closure and cheek bulging. Upon admission, her condition progressed to difficulty opening her mouth and bilateral facial nerve paralysis. She had no prior history of hypertension, diabetes, or coronary artery disease.

Vital signs were within normal limits: temperature 36.2°C, heart rate 78 beats/min, respiration 20 breaths/min, and blood pressure 125/88 mmHg. Cardiopulmonary and abdominal examinations revealed no significant abnormalities. Neurological examination showed shallow left frontal lines and nasolabial sulcus, with bilateral cheek puffing weakness. Eye movements were normal. Muscle strength was normal in the upper limbs, but lower limb strength, initially graded as 5- on the MMT scale, progressively declined to 3+. Muscle tone in all limbs was normal. Tendon reflexes were normal in the upper limbs, absent in the lower limbs, and ankle reflexes were weakened. Babinski's sign was negative, and coordination tests were stable and accurate. Sensory examination revealed bilateral hyperalgesia of the face and ankles. Meningeal irritation signs were negative, and neck movement was unrestricted.

Laboratory investigations showed an elevated monocyte percentage (11.8%) and absolute monocyte count (0.8×10^9/L). Serum amyloid A levels were slightly elevated (12.28 mg/L). Epstein–Barr virus (EBV) tests showed positive viral capsid antigen IgM (3.82 AU/mL) and nuclear antigen IgG (84.34 AU/mL). Respiratory virus antibody testing revealed positive influenza B IgG antibodies. Electrolytes, liver and kidney function, folate, thyroid function, and other routine tests were normal.

Electromyography and nerve conduction studies (NCS) revealed significant abnormalities consistent with GBS. Motor nerve testing showed prolonged distal latency in the bilateral tibial and peroneal nerves, reduced compound muscle action potential (CMAP) amplitudes in the lower limbs, slowed motor conduction velocities in the bilateral tibial, peroneal, and median nerves, and conduction blocks in the peroneal and tibial nerves. F-wave analysis revealed absent F-waves in the lower limb nerves and delayed F-wave latency in the median nerve. Sensory nerve testing demonstrated absent sensory nerve action potentials in the sural and ulnar nerves. Facial nerve studies revealed prolonged distal latency and reduced CMAP amplitudes bilaterally. These findings are consistent with acute demyelinating polyradiculoneuropathy (AIDP), the most common form of GBS, characterized by significant motor and sensory nerve involvement. The presence of bilateral facial nerve damage further supports the atypical presentation observed in this case.

After admission, a lumbar puncture was performed: pressure was 95 mmH_2O (1 mmH_2O = 0.98 kPa), cerebrospinal fluid (CSF) was clear, CSF examination: protein was 1.58 g/L (0 – 0.4 g/L), cell count (CSF) was 5×10^6/L, and there was a clear protein-cell separation. The CSF levels of IgG, IgA, and IgM were 96.0 mg/L (0 – 34 mg/L), 13.7 mg/L (0 – 5 mg/L), and 11.7 mg/L (0 – 1.3 mg/L), respectively.

CSF ganglioside antibody 24 items were analyzed by immunoblotting, and sulfatides-IgG was 533.85 (+) (0 – 50) and the rest of the antibodies were negative (Table 1 and Figure 1). Sample testing was outsourced to ADICON Holdings Limited. For Western blot, qTouch Western Blot Imager (RWD Life Science) was used as the detection method.

Magnetic resonance imaging (MRI) of the pelvis and lumbar spine revealed mild disc herniation and disc degeneration at L4-L5 and L5-S1. Cranial, neck, and chest MRI showed no significant abnormalities in the brain but mild atherosclerosis of the posterior cerebral arteries and a thin right anterior cerebral artery, likely a congenital variant. Cervical spine imaging showed mild herniation of intervertebral discs at C4-C5 and C5-C6, whereas thoracic spine imaging revealed disc degeneration at T4-T5, T5-T6, and T6-T7, along with partial fusion of T6 and T7 vertebral bodies.

Table 1. Index of 24 items of ganglioside antibodies in patients (in English)

Index	Results
Sulfatides-IgM (CSF)	Negative (<50)
Sulfatides-IgG (CSF)	Positive (533.85)
GM1-IgM (CSF)	Negative (<50)
GM1-IgG (CSF)	Negative (<50)
GM2-IgM (CSF)	Negative (<50)
GM2-IgG (CSF)	Negative (<50)
GM3-IgM (CSF)	Negative (<50)
GM3-IgG (CSF)	Negative (<50)
GM4-IgM (CSF)	Negative (<50)
GM4-IgG (CSF)	Negative (<50)
GD1a-IgM (CSF)	Negative (<50)
GD1a-IgG (CSF)	Negative (<50)
GD1b-IgM (CSF)	Negative (<50)
GD1b-IgG (CSF)	Negative (<50)
GD2-IgM (CSF)	Negative (<50)
GD2-IgG (CSF)	Negative (<50)
GD3-IgM (CSF)	Negative (<50)
GD3-IgG (CSF)	Negative (<50)
GT1a-IgM (CSF)	Negative (<50)
GT1a-IgG (CSF)	Negative (<50)
GT1b-IgM (CSF)	Negative (<50)
GT1b-IgG (CSF)	Negative (<50)
GQ1b-IgM (CSF)	Negative (<50)
GQ1b-IgG (CSF)	Negative (<50)
Outcome	**Interpretation**
<50	- Negative
50 – <350	(+) Suspected positive
350 – <650	+ Weak positive
650 – <950	++ Moderately positive
≥950	+++ Strong positive

Abbreviation: CSF: Cerebrospinal fluid; Ig: Immunoglobulin.

2.1. Diagnosis

The patient was diagnosed with anti-sulfatide antibody-positive GBS (paraplegia with facial nerve palsy type), cervical disc herniation, and lumbar disc herniation.

2.2. Treatment and outcome

The patient received intravenous Ig (0.4 g/kg) for 5 days, intramuscular nerve growth factor, methylcobalamin (1 mg twice daily), Vitamin B6 (0.2 g twice daily), pregabalin, Vitamin B1, and ibuprofen. After 14 days, the patient's lower limb weakness and facial nerve paralysis improved significantly. She could walk in a straight line, had a natural facial expression, fluent speech, and reduced numbness and pain. A follow-up 2 weeks later confirmed a stable condition with no recurrence.

3. Discussion

GBS is an autoimmune-mediated acute inflammatory peripheral neuropathy that often develops after an infection. Studies have reported that up to 35% of GBS patients in the IGOS study had a history of upper respiratory tract infection.[5] Common triggers include EBV, influenza B virus, and cytomegalovirus.[6] GBS usually presents as AIDP or acute motor axonal neuropathy (AMAN).

In this case, the patient had a history of infection with EBV and influenza B. Initial symptoms were lumbosacral pain that progressed to lower extremity pain, numbness, weakness, and loss of tendon reflexes. Symptoms progressed downward with bilateral facial nerve palsies. Despite lower extremity weakness, upper extremity strength was normal. Electrophysiological studies showed demyelinating changes such as prolonged motor nerve conduction latency, slowed conduction velocity, and conduction block. CSF analysis showed protein-cell dissociation and positive anti-sulfate IgG antibodies, confirming a GBS spectrum disorder.

The final diagnosis was anti-sulfate-positive GBS (paraplegia with bilateral facial nerve palsy), a rare form that accounts for 5.2% of GBS cases.[7] Initially resembling sciatica, lumbar spine MRI showed mild L4-L5 and L5-S1 disc herniation but no nerve compression. This atypical presentation emphasizes the importance of considering GBS in cases resembling sciatica, especially in cases with similar serological and electrophysiological findings.

The patient's GBS was treated with intravenous Ig, supporting immune modulation and recovery.[4] Adjunct therapies, including nerve growth factor, methylcobalamin, Vitamins B1 and B6, and pregabalin, promoted axonal repair, myelin regeneration, and pain relief.[8] This comprehensive approach facilitated significant improvement in lower limb strength and facial nerve function.

Previous studies on paraplegic GBS primarily focused on anti-ganglioside antibodies (AGAs) (Table 2). van den Berg *et al.*[9] reported 40 paraplegic GBS cases among 490 patients, with four positive for AGAs. Wakerley *et al.*[10] found one case among 103 patients; whereas Kim *et al.*[11] identified three cases among 13 patients. Anti-sulfatide antibody positivity remains unreported.



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检测结果统计

指标	检测结果	检测方法	参考区间
Sulfatides-IgM(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
Sulfatides-IgG(脑脊液)	阳性（533.85）	免疫印迹法	阴性<50
GM1-IgM(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GM1-IgG(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GM2-IgM(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GM2-IgG(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GM3-IgM(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GM3-IgG(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GM4-IgM(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GM4-IgG(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GD1a-IgM(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GD1a-IgG(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GD1b-IgM(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GD1b-IgG(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GD2-IgM(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GD2-IgG(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GD3-IgM(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GD3-IgG(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GT1a-IgM(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GT1a-IgG(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GT1b-IgM(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GT1b-IgG(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GQ1b-IgM(脑脊液)	阴性（<50）	免疫印迹法	阴性<50
GQ1b-IgG(脑脊液)	阴性（<50）	免疫印迹法	阴性<50

结果判读	免疫印迹法	
注一：	血清或脑脊液	
结果		判读
<50	-	阴性
50 - <350	(+)	疑似阳性
350 - <650	+	弱阳性
650 - <950	++	中阳性
≥950	+++	强阳性

注二：病情变化及治疗的影响，病人体内的抗体(阴阳性或浓度)处于动态变化中，可导致不同时间检测的结果出现差异。

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Figure 1. Index of 24 tests for ganglioside antibodies in cerebrospinal fluid (in Chinese)

These studies highlight the limitations of using AGAs for diagnosing GBS, as many patients test negative for AGAs. While epidemiologic studies suggest ganglioside-based drugs do not increase GBS risk, case reports indicate otherwise.[18,19]

The emergence of anti-sulfatide antibodies is particularly significant for GBS patients negative for AGAs. Sulfatide, an acidic glycolipid abundant in peripheral nerve tissues, is crucial for maintaining nerve sheath structure, modulating impulses, and transmitting signals.[20]

Elevated sulfatide antibodies are linked to widened myelin membrane gaps, demyelination, and axonal degeneration.[21] Studies confirm that sulfatide deficiency destabilizes myelin proteins, impairs myelin-axon interactions, and reduces myelin stability.[22,23]

Anti-sulfatide antibodies are associated with the AMAN subtype of GBS and are also seen in chronic demyelinating polyneuropathy and other neuropathies. Recognizing sulfatide antibodies provides valuable insights into GBS pathogenesis, particularly in patients without AGA positivity.

A PubMed search for "sulfatide" and "GBS" revealed four matching articles, summarized in Table 3.

Sulfatide has significant implications for diagnosing and treating central nervous system disorders. Qiu *et al.*[28] found that sulfatide deficiency drives AD-like neuroinflammation. Zimmer *et al.*[29] demonstrated that sulfatide supplementation can decrease Aβ production and is a potential therapeutic target for AD pathogenesis. Sulfatide antibody positivity has also been observed in multiple sclerosis (MS), with Hamatani and Kondo[30]

Table 2. Literature review of paraplegic Guillain–Barré syndrome cases and anti-ganglioside antibody findings

Literature	Sex/Age	Precursor symptoms	Symptoms	Sensory abnormalities	Cranial or autonomic nerve damage manifestations	Anti-ganglioside antibodies
Scheidl *et al.*[12]	F/54	Upper respiratory tract infection	Weakness in both lower limbs	Numbness and pain in all limbs	NA	NA
Vishnuram *et al.*[13]	M/35	Upper respiratory tract infection	Weakness in both lower limbs	None	Nausea, vomiting	NA
Mori *et al.*[14]	M/35	Hand, foot, and mouth disease	Weakness in both lower limbs	Numbness in limbs, pain in neck, back, and waist	Left facial nerve paralysis	Antibody negative
Bigaut *et al.*[15]	M/48	Upper respiratory tract infection	Flaccid paraparesis	Sensory abnormalities in limbs, pain in lower limbs	Right facial nerve paralysis	Antibody negative
Börü *et al.*[16]	M/42	Upper respiratory tract infection	Weakness in both lower limbs	NA	Nausea, vomiting, urinary retention	NA
Kimachi *et al.*[17]	F/79	Upper respiratory tract infection	Weakness in both lower limbs	Pain in both lower limbs	None	GM1 AGA and GD1b AGA positive

Abbreviation: AGA: Anti-ganglioside antibodies.

Table 3. Summary of Guillain–Barré syndrome variants and related antibody findings including the present case

Literature	Sex/ Age	Precursor symptoms	Symptoms	Sensory abnormalities	Cranial or autonomic nerve damage manifestations	Anti-ganglioside antibodies
Zhao, *et al.*[24]	M/20	Upper respiratory tract infection	Ophthalmoplegia, ataxia, weakness in the right limbs	Anti-GQ1b antibody positive with MFS/GBS overlapped syndrome with diplopia and hemiplegia onset	GQ1b-IgG AGA, G-IgG AGA	Anti-sulfatide IgG antibodies
Liu *et al.*[25]	F/68	No upper respiratory tract infection	Weakness and numbness in limbs, neck pain, urinary and fecal retention	Variant of GBS with anti-sulfatide antibody positivity and spinal cord involvement	NA	Anti-sulfatide IgG antibodies
Luan *et al.*[26]	F/77	NA	Weakness in limbs, dysarthria	Recurrent GBS presenting as pharyngeal-cervical-brachial variant with three species of ganglioside antibodies	GD1a AGA, GT1a AGA	Anti-sulfatide antibody positive
Souayah *et al.*[27]	M/38	HIV infection	General weakness and fever	GBS associated with untreated end-stage AIDS	NA	Anti-sulfatide IgG and lgM antibodies
Present case	F/42	EBV and Influenza B infection	Paraplegia, bilateral facial nerve palsy	GBS with paraplegia and bilateral facial nerve palsy	Bilateral facial nerve paralysis, tongue numbness	Anti-sulfatide IgG antibodies

Abbreviations: AIDS: Acquired immunodeficiency syndrome; EBV: Epstein–Barr virus; GBS: Guillain–Barré syndrome; Ig: Immunoglobulin; MFS: Miller Fisher syndrome.

showing that sulfatide can treat MS by modulating T-cell activation. Sulfatide supplementation in the hippocampus attenuated oxidative brain damage,[31] and adult-onset sulfatide depletion caused axonal function loss and irreversible neuronal function loss.[20]

4. Conclusion

This case underscores the potential role of sulfatide supplementation in treating neurological disorders, including anti-sulfatide antibody-positive GBS. Since nerve growth factor increases ceramide release and sulfatide is synthesized from ceramides,[23,32] adhering to treatment guidelines that incorporate nerve growth factor may enhance sulfatide synthesis and improve patient outcomes. Further studies are needed to explore optimal levels of supplemental sulfatide therapy and confirm its therapeutic benefits.

As research advances, the pathophysiologic mechanisms of sulfatide in GBS are becoming clearer. Future studies should focus on determining the minimum sulfatide levels required for therapeutic efficacy, aiding in the development of targeted treatments for this rare GBS variant. This case highlights the importance of continued research to refine diagnostic criteria and therapeutic approaches, enabling clinicians to better manage similar presentations and improve patient outcomes.

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Conflict of interest

The authors declare no competing interests.

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Ethics approval and consent to participate

Ethical approval was obtained from the Affiliated Hospital of Weifang Medical College, China (approval no.: WYFY-2024-QT-019). Patient gave written consent to participate in this study.

Consent for publication

Patient gave verbal consent to publish her data in this study.

Availability of data

Not applicable.

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CASE REPORT

Refractory musicogenic epilepsy and a surgical cure guided by metabolic neuroimaging: A case report

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Abstract

A 40-year-old female patient with refractory musicogenic epilepsy and non-lesional magnetic resonance imaging neuroimaging is highlighted in this report. A right temporal lobectomy was necessary and sufficient to effect a durable surgical cure without recurrence for over approximately 18 years in follow-up while remaining off seizure medications for the prior 16 years. Our case report delineates that this patient was cured with a surgical resection guided by metabolic neuroimaging. This patient was evaluated for cerebral localization of seizure onsets to guide further therapy in an epilepsy center and the evaluation for localization of seizure onset identified that Ictal single photon emission computed tomography (SPECT) imaging identified an approximately 25% increase in blood flow or metabolism in the right temporal lobe during seizures compared with the baseline. Seizure semiology and electroencephalography localization were concordant with SPECT localization.

Keywords: Intractable epilepsy; Metabolic neuroimaging; Non-lesional epilepsy; Surgery

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1. Introduction

Although musicogenic epilepsy has been previously described, this unique case report identifies how a long-lasting and a durable surgical cure of approximately 18 years maintained subsequent to an anterior temporal lobectomy, which was decided under the guidance of ictal single photon emission computed tomography (SPECT) metabolic neuroimaging in a patient with a non-lesional magnetic resonance imaging (MRI). An overview of the literature and discussion of anatomic localization and a brief contemporary overview of musicogenic epilepsy are highlighted in the article. This paper also highlights current literature involving peri-ictal characterization and techniques through source imaging and functional neuroimaging techniques such as MRI (functional MRI [fMRI]) with adjunctive invasive electroencephalography (EEG) monitoring.

2. Case presentation

Herein, we report a 40-year-old right-handed female patient who had seizures in response to hearing music – in particular music from popular films and other tonal-type music. The music-induced seizures include loss of awareness and chewing and fumbling automatisms. This patient is the product of a normal full-term delivery and was the firstborn. There were no febrile convulsions as a youngster and had normal early neurodevelopment. She had the first seizure around the onset of menarche (approximately age 12) and had seizures subsequently since then. In general, the seizures begin with a rising epigastric sensation or simply like a feeling in her abdomen that is difficult to describe followed by loss of consciousness. The patient claimed that she often can understand what is being said around her, and the event lasts about 30 s and after the epileptic episode, she will become sweaty and have the urge to urinate. Sometimes, she is drowsy, and these events can happen up to several times per month. Loud dramatic music such as in "Batman" and especially "Harry Potter" films seem to precipitate these events, particularly perimenstrually. She could not operate motor vehicles and indicated that her memory may be deteriorating over time. She had occasionally generalized tonic-clonic seizures.

Her MRI revealed non-lesional findings. EEG localized the seizure onset to the right temporal lobe and identified the semiology as focal seizures: complex partial seizures or focal unaware seizures with focal to bilateral or secondary generalization. Ictal spectroscopy done within seconds of onset of a seizure identified the regions of the brain experiencing approximately 25% increased blood flow compared to the follow-up scan, which served as the baseline. The patient ultimately had a right temporal lobectomy and has not had seizures in approximately 20 years postoperatively and of note, medications were tapered completely approximately 18 years previously. Pathology showed non-specific gliosis and perivascular lymphocytic aggregates and some hemosiderin-containing macrophages, which were generally non-lesional. It is important to note that her past medical history included hypertension, diabetes, and previous hysterectomy. She had attempted monotherapies of levetiracetam, lamotrigine, phenytoin, valproic acid, carbamazepine, and phenobarbital in varied combinations; however, these regimens failed in terms of therapeutic efficacy.

Metabolic imaging that localized seizure onsets facilitated a durable cure over an approximately 18-year period and the patient has remained off all seizure medications 2 years after the surgery. Figure 1 denotes panels of the ictal and interictal metabolic neuroimaging. Figure 2 highlights images of pre- and post-operative 1.5 T MRI brain neuroimaging, and Figure 3 shows successive panels of EEG during a typical musicogenic seizure.

3. Discussion: Reflex epilepsy, epilepsy, and metabolic imaging

Reflex epilepsies are provoked by either external stimuli or internal mental processes and there exists literature on the mechanisms and underlying genetics involved.[1-7] Patients may have coexisting spontaneous seizures in addition to those provoked by certain stimuli, and the seizures may present as focal or generalized.[1-7] While photosensitive epilepsy is the most common reflex epilepsy and has been extensively reviewed, reflex epilepsies have been noted with reading, hearing music, somatosensory stimulation, praxis, or unique experiences such as being immersed in water.[1-7] It has been reported that music can provoke an ictal EEG showing rhythmic 4 – 7 Hz theta activities that originate in the right temporal lobe, and subtraction ictal and interictal SPECT co-registered to MRI (SISCOM) shows ictal hyperperfusion in the right insula, amygdala, hippocampal head, and anterior temporal lobe, while 18-flurodeoxyglucose-positive emission tomography (18-FDG-PET) shows interictal hypometabolism in the same brain regions.[3,7] The literature supports metabolic imaging as a useful way of localizing seizure onset as part of the clinical evaluation of patients.[8-11]

Our case involved SPECT showing involvement of anterior neocortex, amygdala, and insula, which are structures are consistent with pathways denoted in prior literature such as in the study by Gelisse *et al.*[3] indicating general right temporal hypometabolism on 18-FDG-PET; in the study by Cho *et al.*[7] which reported that SISCOM imaging revealed ictal hyperperfusion in the right insula, amygdala, hippocampal head, and anterior temporal lobe, while interictal FDG-PET showed hypometabolism in these same; and in a more recent study by Bratu *et al.*[10] indicating the neocortex as a predominantly noted localization for a GAD65-mediated autoimmune antibody process causing temporal lobe epilepsy. In Stern's review of the literature, he speculates that the precise localization of musicogenic epilepsy may be heterogenous due to the varied localization techniques used and that the circuitry may appear heterogenous due to an individual's unique response, unique processing, and ultimately emotional experience to music which pathways may be differentially highlighted on testing and are likely to be denoted and discerned generally to be downstream from primary auditory cortex or Heschl's gyrus.[11] Musicogenic epilepsy cases may have predominantly been detected through non-lesional imaging, and authors such as Stern,[11] Kaplan,[12] and others such as Goldman *et al.*[16] speculated that other tests

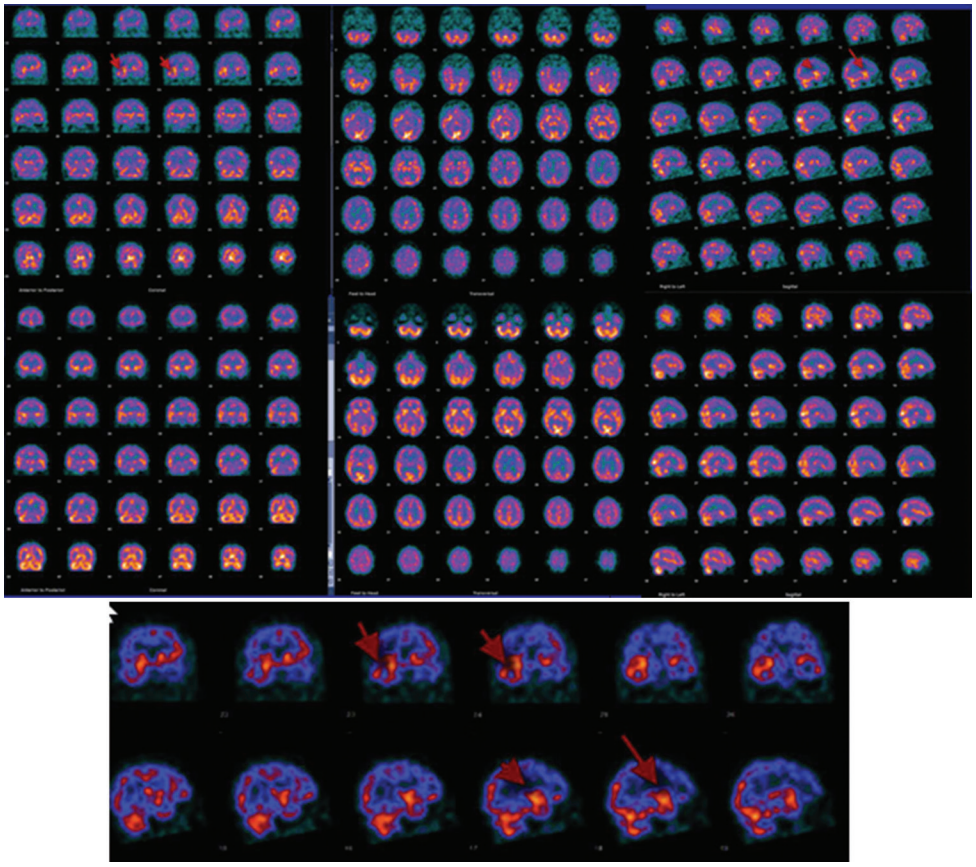


Figure 1. Dataset of ictal and interictal neurolytic Tc99 metabolic SPECT neuroimaging. Top panels indicate blood flow distribution of a typical seizure/ictal event. Arrows denote regions of an approximately 25% increase in blood flow compared to the baseline (middle panels) in the right temporal lobe involving the anterior neocortex, amygdala, and insula predominantly. Middle panels show interictal metabolism or blood flow. Double panels at the bottom delineate the signature highlighted sections showing the most significant changes in blood flow during a seizure.
Abbreviation: SPECT: Single photon emission computed tomography.

such as interictal characterization using source magnetic imaging might be helpful along with fMRI analyses in localizing ictal onset zones which might be resected for effecting a cure. Furthermore, in general, there exist only small number of successful case series or reports with some showing inconsistent or even bilateral lateralization and early post-operative seizures if limited resection is pursued even after invasive EEG monitoring identifies such discrete onset zines and therefore this literature indicates that a potential wider resection might be required to eradicate a sufficient amount of epileptogenic network for a durable cure. However, Stern speculates that the essential portion of the anatomical substrate involved in the semiology of musicogenic epilepsy is predominantly the limbic networks in the anterior mesial temporal lobes, and likely it is the subsequently occurring network involved in the cognitive processing distal to the primary limbic networks involving frontal or association areas and or neocortical regions that lead to the phenomenology as temporal resections might offer seizure eradication as more proximal input to the portions of the network that may involve affective or emotional or other processing.[11-17]

While the literature may support an evaluation using metabolic neuroimaging among other techniques and ictal and interictal testing with functional neuroimaging, we speculate that identifying a case of musicogenic epilepsy in which the ultimately major clinically relevant primary epileptic zone or region was localized by the metabolic imaging which was concordant with ictal EEG data in a patient who had a non-lesional MRI that resulted in a surgical cure with an extended multi-year follow-up is a rare but notable finding. Our case indicates that although there is a potentially more widespread and distal neural network involved in the pathophysiology of musicogenic reflex epilepsy as supported in the literature cited above which highlights source localization techniques, fMRI, and invasive EEG monitoring, a right temporal lobectomy – guided predominantly by the metabolic neuroimaging – was the necessary and sufficient procedure that effected

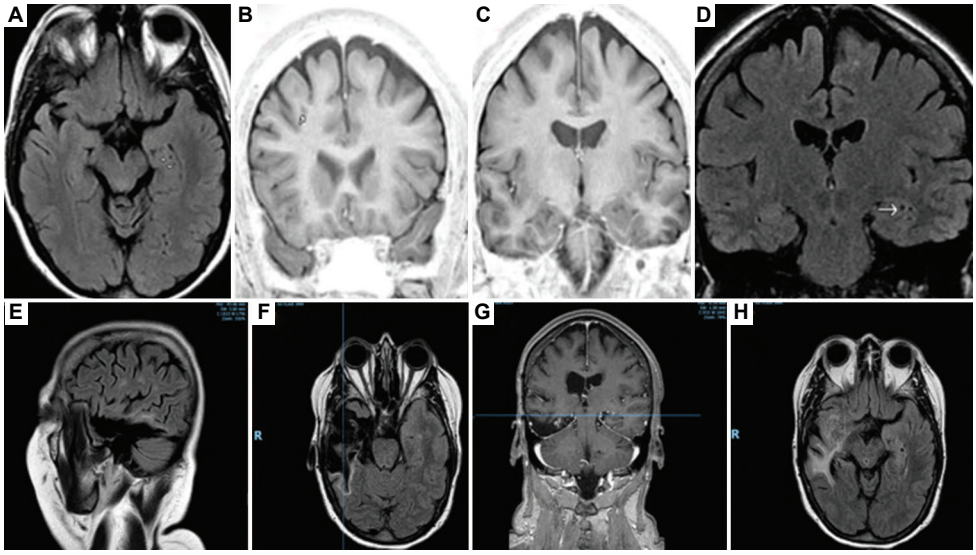


Figure 2. Highlighted panels of MRI neuroimaging. Top panels show images from preoperative imaging. (A) 1.5 T MRI A-Axial flair. (B) Coronal inversion recovery image through temporal lobe. (C) Coronal inversion recovery image at level of amygdala. (D) Coronal flair at the level of the bilateral hippocampi. Arrow in (B) points to non-specific gliosis by radiology, and arrow in (D) is consistent with a prominent Virchow–Robin space. Bottom panels are highlighted panels of post-operative 1.5 T MRI 15 years postoperatively. (E) Sagittal flair and (F)-axial flair at the level of the pons. (G) Coronal T1 post gadolinium section. (H) Axial flair at the level of the midbrain. The bottom panels denote probably nonspecific post-operative posterior temporal gliosis.
Abbreviations: IR: Infrared; MRI: Magnetic resonance imaging.

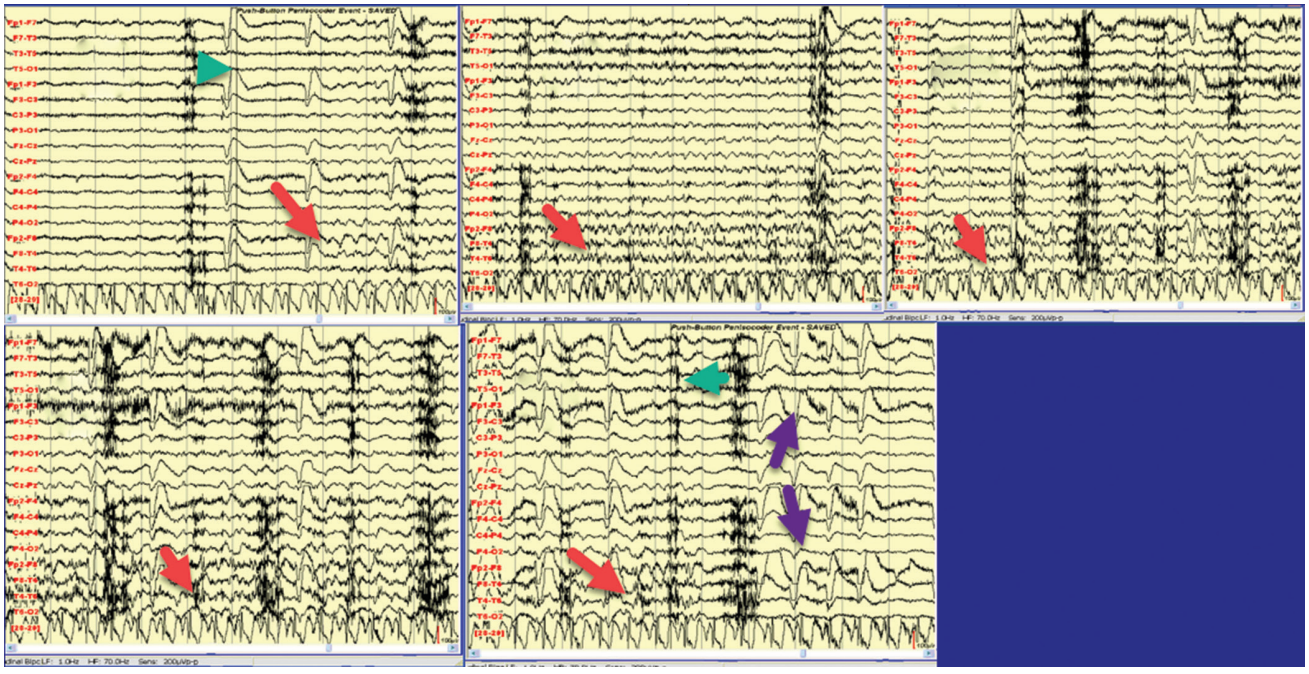


Figure 3. Successive EEG panels during a musicogenic seizure. Green arrows mark the onset and offset of the event. Red arrows denote evolving rhythmic theta frequencies over the right temporal region. Purple arrows identify post-seizure attenuation and the postictal phase.
Abbreviation: EEG: Electroencephalography.

a surgical cure as outlined in this case report. Given the above literature review, we speculate that although invasive investigation with intracranial EEG may have been opined or pursued to delineate more discrete localization, which might have resulted in a more limited resection or focal topectomy, such an approach might not have resulted in a durable cure for such an extended follow-up period.

4. Conclusion

Although neuronal connectivity and pathways for musicogenic epilepsy may not be confined to the temporal region, our report highlights that a temporal lobectomy produced an 18-year durable cure. Our report is additionally notable since the literature on this topic may not have such an extensive follow-up period studied and in addition, early post-operative seizure recurrence has been noted in other reports which intended to effect more discrete resections within such a network guided by other techniques such as invasive EEG monitoring as outlined in the discussion.

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Conflict of interest

The authors have no conflict of interests to declare.

Author contributions

Conceptualization: All authors
Formal analysis: All authors
Investigation: All authors
Methodology: All authors
Writing – original draft: Joel M. Oster
Writing – review & editing: All authors

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Availability of data

Not applicable.

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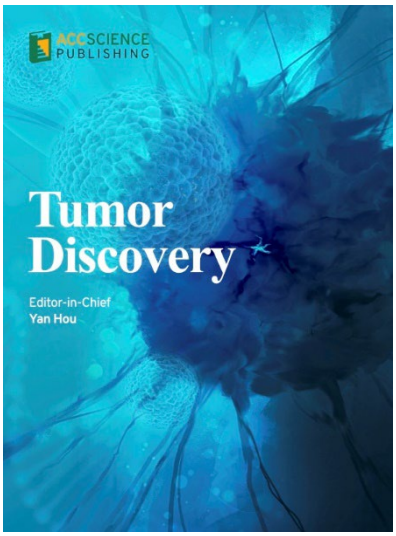
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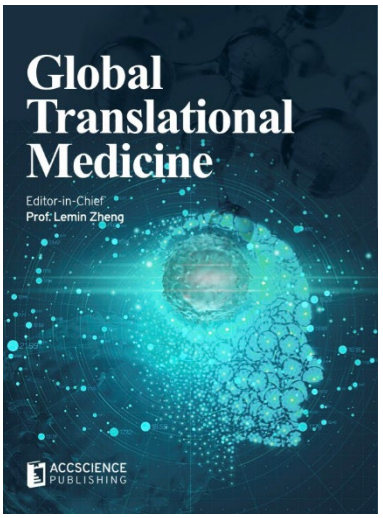


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