ADVANCES IN DIAGNOSIS AND TREATMENT OF TBI-INDUCED NEURODEGENERATION AND COGNITIVE DEFICITS

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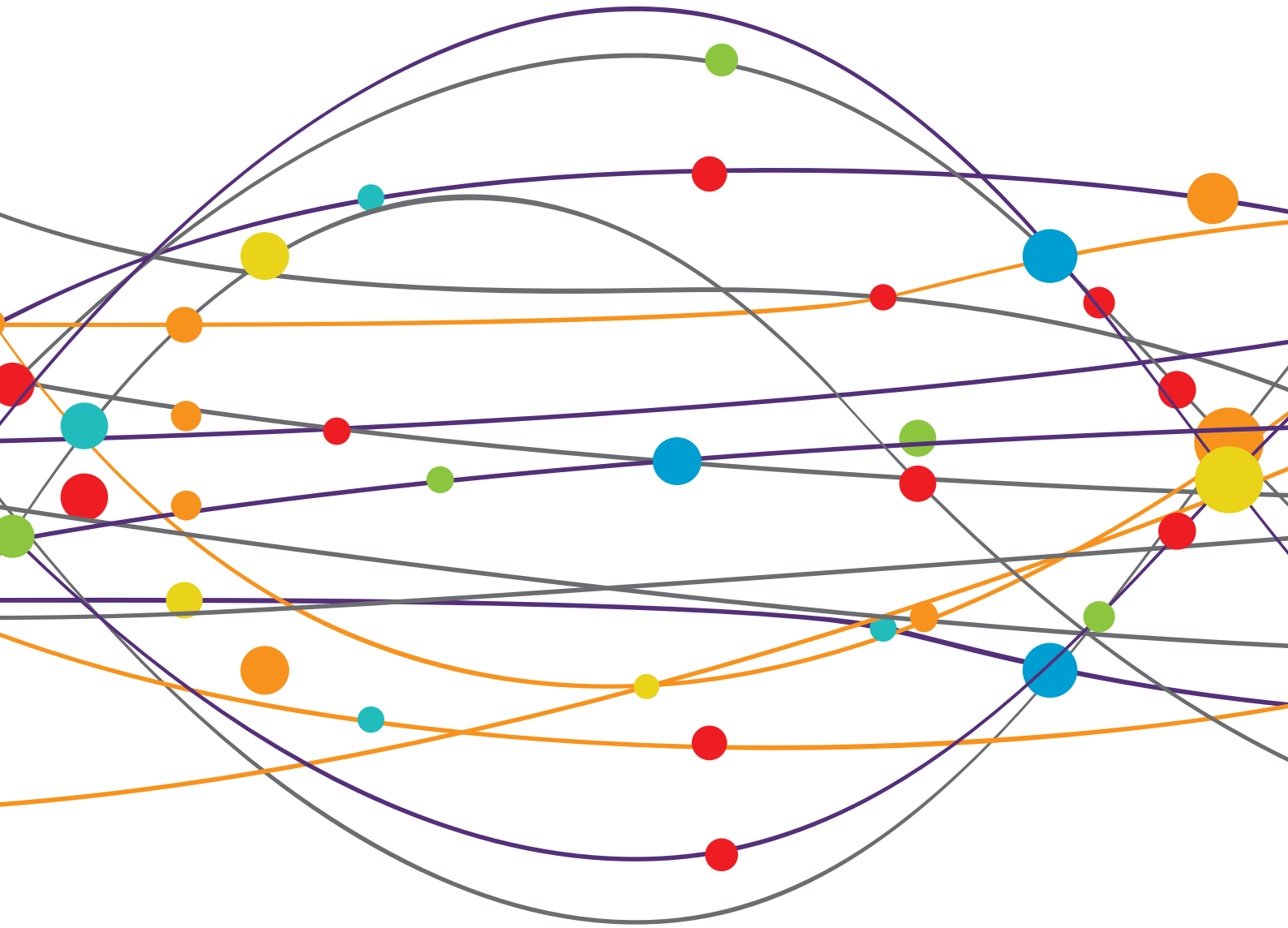

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ADVANCES IN DIAGNOSIS AND TREATMENT OF TBI-INDUCED NEURODEGENERATION AND COGNITIVE DEFICITS

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EDITORIAL
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Editorial: Evidence-Based Anti-Inflammatory, Anti-Hypoperfusion and Anti-Anxiety/Insomnia Therapies Show Promises for TBI-Induced Post-Traumatic Symptoms and Cognitive Deficits: Advances in Diagnosis and Treatment of TBI-Induced Neurodegeneration and Cognitive Deficits

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Editorial on the Research Topic

Advances in Diagnosis and Treatment of TBI-Induced Neurodegeneration and Cognitive Deficits

A progressive series of chronic microhemorrhages and inflammatory insults on the cerebrovascular system and neural-network integrity can lead to significant neurological and cognitive impairments following mild-to-moderate traumatic brain injury (mTBI). Evidence-based effective treatment is needed to stop this progression. Seventeen studies in this Research Topic (4 reviews, 2 prospective, 4 animal/cellular model studies, 5 clinical studies, and 2 clinical case reports) have investigated the mechanisms, diagnosis, and novel therapies of TBI-induced neuropathology and cognitive impairments. Activated inflammatory microglia, elevated Inflammatory cytokines (specifically CCL2 and IL-1β), oxidative stress, blood-brain barrier (BBB) disintegration, microvascular inflammatory injury-induced de-coupling between high metabolic demands and compromised cerebral blood flow (CBF)/perfusion, and brain network senescence/dysfunction associated with hyper-phosphorylated tau tangling appear to be the important pathological mechanisms following TBI. These disruptions of normal brain function underlie the disorders of consciousness, insomnia, depression, and neurological and cognitive impairments that follow mTBI. These pathological changes can now be determined by innovative assays and neuroimaging technologies. The advances in this field are leading to innovative treatments for the disorders which follow mTBI.

Developing approaches include intraneuroendoscopic removal of subdural hematomas, protecting BBB integrity by methylene blue or by medicinal herbs, controlling neuroinflammation by endocannabinoids, improving CBF/hypoperfusion by photobiomodulation or by executive function training (including computerized training and playing mahjong) or by transcranial direct current stimulation (tDCS), improving insomnia by cognitive-behavioral therapy. All of these approaches appear to be promising interventions to improve post-traumatic symptoms and cognitive deficits associated with mTBI.

As long-term repetitive sub-concussive impacts induce subtle neuromotor dysfunction in animals, similarly, clinicopathological changes and dementia in a 93-year-old street boxer are likely due to the brain concussions that occurred many years before. Accordingly, early diagnosis and treatment could mitigate the damage from repeated sub-concussive impact-induced chronic brain inflammation and resulting subjective cognitive decline (SCD) that had occurred in early life. Measures are developing which will likely provide effective prophylactic approaches that prevent dementia.

SCD, now recognized as an occasional preclinical sign of Alzheimer's disease (AD) and dementia, may be common in people with a history of TBI. Si et al. reviewed recent progress in SCD research and its relevance to TBI-induced cognitive deficits. SCD is characterized by early self-perceptive cognitive decline before the detection by objective tests. If identified, SCD can be treated/reversed more easily than later stages of progression from mild cognitive impairment (MCI) to dementia.

Sun et al. showed that serum CCL2 level was elevated over 3 months and was associated with the severity of post-concussion symptoms in 52 patients with acute mTBI compared to 54 healthy subjects, whereas elevated IL-1β was associated with worsened working memory in the acute phase of TBI. Thus, elevated inflammatory CCL2 and IL-1β could be predictors of post-concussion symptoms and cognitive outcomes of mTBI.

By studying inflammatory factors, Shang et al. showed that IL-1β effectively induced cellular senescence in rat astrocytes that was accompanied by increased total and phosphorylated tau. Similarly, both oligomerized amyloid β (Aβ) and H_2O_2 induced cellular senescence in rat astrocytes by NLRP3-mediated IL-1β secretion. They proposed that adverse stimuli-induced NLRP3 activation and IL-1β production are potentially diagnostic biomarkers and therapeutic targets for AD/dementia.

Shang et al. reported reduced serum miR-451 levels in patients with intracerebral hemorrhage (ICH) compared to healthy controls. They further evaluated the role of miR-144/451 cluster in ICH mice model. Deletion of miR-144/451 cluster exacerbated neurological deficits and brain edema, significantly promoted TNF-α and IL-1β secretion and oxidative stress in the perihematomal region of knockout mice compared with wild-type ICH animals, supporting a neuroprotective role of miR 144/451against ICH-induced inflammation and oxidative stress.

Yang et al. presented clinicopathological evidence of the coexistence of AD with hyper-phosphorylated tau-pathology, **c**hronic traumatic encephalopathy (CTE), dementia with Lewy bodies, and hippocampal sclerosis with TDP-43 pathology in a 93-year-old former street boxer with a pre-mortem diagnosis of severe dementia. CTE is a tauopathy in which multifocal perivascular phosphorylated tau aggregates accumulate in astrocytes, neurons, and neurites at the depths of the cortical sulci. These results suggest that early repetitive sub-concussive impacts may trigger pre-mortem pathologies of CTE, AD, and dementia.

Lavender et al. report that highly repetitive sub-concussive impact with a lightweight drop (25 g) onto anesthetized female rats for 12 weeks induced detectable/subtle neuromotor dysfunction i.e., increased foot slips in beam-walk and rotarod tests. Thus, long-term repetitive sub-concussive impacts may also be a mechanism underlying clinicopathological changes of the 93-year-old street boxer reported by Yang et al..

Tanaka et al. reviewed how modulation of the endocannabinoids (eCBs) system (composed of cannabinoid receptors, ligands, and metabolic/biosynthetic enzymes) could facilitate the homeostatic microglia to adopt either the (M1) state, which secretes mediators of the proinflammatory response, or to the (M2) state, which mediates the resolution of neuroinflammation and tissue repair/remodeling in neuropathological conditions. They propose that cannabinoid 2 receptor signaling pathway plays a critical role in shifting the microglia from the pro-neuroinflammatory (M1) state to the anti-inflammatory/neuroprotective (M2) state.

Du et al. showed in a non-randomized controlled trial that the intraneuroendoscopic technique (INET) is significantly better than traditional burr hole drainage (BHD) in the treatment of subacute and chronic septal subdural hematoma in terms of hematoma recurrence rate and subdural drainage tube (SDT) placement duration at 6-months post-operative follow-up.

Chronic microvascular injury/microbleeding following TBI may lead to inflammation and vascular cognitive impairment (VCI) which is difficult to diagnose. In their review paper, Wang et al. proposed MRI as the first choice for suspected VCI to evaluate brain atrophy, infarction, white matter hyperintensity, and hemorrhage. Resting-state functional magnetic resonance imaging (rs-fMRI) can detect spontaneous brain functional activity, probe the pathogenesis of VCI in-depth and provide a reference for early diagnosis and prognostic evaluation.

Shen et al. demonstrated that methylene blue (MB) treatment can reverse mitochondrial dysfunction following oxygen glucose deprivation/reoxygenation (OGD) in PC12 cells, reduce neuronal apoptosis and improve blood-brain barrier (BBB) integrity in a fluid percussion TBI mouse model. MB also inhibited ROS production, stabilized neuronal mitochondrial membrane potential (MMP), increased ATP production, and preserved brain functions following TBI as demonstrated by Morris water maze, rotarod, and modified Neurological Severity Score (mNSS) tests.

Reduced cerebral blood flow (CBF) and perfusion may underlie persistent post-traumatic symptoms of mild-moderate TBI, but these parameters might be difficult to measure. Quinn et al. applied pseudo-continuous arterial spin labeling (pCASL) magnetic resonance imaging techniques in measuring CBF in 24 subjects with mild and moderate TBI before and after 10 days of computerized executive function training (CEFT)

combined with active or sham anodal transcranial direct current stimulation (tDCS). Robust improvements in depression, anxiety, attention, and executive function were observed in both active and sham tDCS groups. tDCS stimulation was associated with static/increased CBF in the right inferior frontal gyrus whereas sham tDCS reduced CBF. Neuropsychological performance and behavioral symptoms, however, were not associated with changes in CBF, probably due to the lack of a control for CEFT. Thus, cerebral perfusion measured with MRI presents a potential pathophysiological target for rehabilitation paradigms of TBI.

Pape et al. showed in a randomized controlled trial that familiar auditory sensory training (a passive auditory stimulation delivered by means of recordings of autobiographical stories narrated by patients' relatives) facilitated changes in neural connectivity in patients with TBI-induced disorder of consciousness. They highlighted a relation between language-related neural networks and improvement in awareness.

In a randomized control trial and by using MoCA, STT, and Functional Activities Questionnaire (FAQ), Zhang et al. showed that playing mahjong for 12 weeks improved the executive function of elderly (74.3 ± 4.3) with mild cognitive impairment (MCI). Since mahjong is a popular 4-people game, further study should determine if mahjong can improve cognitive deficits in people with mild TBI.

Up to 50% of TBI patients suffer from insomnia which may underlie chronic inflammation and is unlikely to subside even after symptoms of mTBI remit. Dietch and Furst provide evidence supporting effectiveness of cognitive-behavioral therapy for insomnia (CBT-I) ($\geq$50% clinical remission rate in general population) and propose more CBT-I studies in patients with mTBI because insomnia is a highly treatable symptom of mTBI that could have broad positive impacts on the recovery of TBI symptoms including cognitive deficits.

Photobiomodulation (PBM) is a therapy that uses red-to-near-infrared (NIR) light to heal injured tissue. Chao et al. reported that 8 weeks of PBM intervention [810-nm light-emitting diodes at 10 or 40 Hz by an intranasal and four transcranial modules targeting nodes of the default mode network (DMN)] increased brain volumes and cerebral perfusion, improved functional connectivity and neuropsychological test scores in a professional hockey player with a history of concussions and chronic symptoms of headaches, mild anxiety, and difficulty concentrating.

By meta-analysis of 36 randomized controlled trials, Lee et al. showed that herbal medicine (HM), more commonly used in China, Asian, and other developing countries as either monotherapy or as adjunctive therapy, are significantly better than conventional treatment (CT) on post-concussion syndrome (dizziness, headache, epilepsy, mental disorder, mild TBI-like symptoms), activities of daily living, neurological dysfunction, and safety profile. However, no differences in traumatic brain edema, posttraumatic hydrocephalus or cognitive dysfunction are found. As most HM studies reported were low-quality due to heterogeneity of the clinical populations, in diagnosis and in the use of active herbal ingredients for TBI treatment, further rigorous standardized RCTs of HB are needed.

As most TBI studies are done in wealthy countries with few data from developing countries, Adamson et al. in collaboration with Enhancing Neuro Imaging Genetics Through Meta-Analysis (ENIGMA) have emphasized the importance of international collaboration and domestic capacity building for innovative research, training clinicians and researchers and databases development to bring developing countries into the international platform for dementia and TBI research.

As we learn more about the evolving neuropathological processes and mechanisms underlying the post-traumatic syndromes following mTBI, better targeted interventions are more likely to be developed. The innovative work published in this Research Topic of Advances in Diagnosis and Treatment of TBI-Induced Neurodegeneration and Cognitive Deficits suggest that evidence-based anti-inflammatory, anti-hypoperfusion, and anti-anxiety/insomnia therapies show promise for TBI-induced post-traumatic symptoms and cognitive deficits.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Conflict of Interest: GX was employed by company Lotus Biotech.com LLC.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Elevated Serum Levels of Inflammation-Related Cytokines in Mild Traumatic Brain Injury Are Associated With Cognitive Performance

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Mild traumatic brain injury (mTBI) is the most common neurological insult and leads to long-lasting cognitive impairments. The immune system modulates brain functions and plays a key role in cognitive deficits, however, the relationship between TBI-induced changes in inflammation-related cytokine levels and cognitive consequences is unclear. This was investigated in the present study in two cohorts of individuals within 1 week of mTBI ($n = 52$, $n = 43$) and 54 matched healthy control subjects. Patients with mTBI were also followed up at 1 and 3 months post-injury. Measures included cognitive assessments and a 9-plex panel of serum cytokines including interleukin (IL)-1β, IL-4, IL-6, IL-8, IL-10, IL-12, chemokine ligand 2 (CCL2), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α). The contribution of cytokine levels to cognitive function was evaluated by multivariate linear regression analysis. The results showed that serum levels of IL-1β, IL-6, and CCL2 were acutely elevated in mTBI patients relative to controls; CCL2 level was remained high over 3 months whereas IL-1β and IL-6 levels were declined by 3 months post-injury. A high level of CCL2 was associated with greater severity of post-concussion symptoms (which survived in the multiple testing correction); elevated IL-1β was associated with worse working memory in acute phase (which failed in correction); and acute high CCL2 level predicted higher information processing speed at 3 months post-injury (which failed in correction). Thus, acute serum cytokine levels are useful for evaluating post-concussion symptoms and predicting cognitive outcome in participants with mTBI.

Keywords: inflammation-related cytokines, post-concussion symptoms, cognitive performance, mild traumatic brain injury, follow-up

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INTRODUCTION

Traumatic brain injury (TBI) is a public health burden and its incidence ranging from 106 to 790 per 1,00,000 people worldwide yearly (1). About 70–90% of TBI are classified as mild TBI (mTBI) (2), which is associated with long-term cognitive impairment including deficits in attention, working memory and executive function. Owing to the heterogeneous pathology of mTBI, there are few effective treatments (3, 4). Guided by preclinical studies partly, therapeutic strategies for mTBI have targeted in protein aggregation, inflammation, metabolic disruption, cell proliferation, and neurotransmitter signaling, but these have had limited success. Clarifying the pathogenic changes underlying cognitive impairment following mTBI can lead to the development of more effective treatment.

Inflammation played a crucial role in cognitive deficits (5–7); for instance, an elevated level of interleukin (IL)−6 was found to be associated with impaired executive function following stroke (8). And has also been implicated in Alzheimer's disease, serum IL-6 is negatively correlated with general cognitive functioning (9). A robust inflammatory response is induced in the injured brain and peripheral circulatory system following a traumatic impact (10), that can persist for many months (11). Inflammation-related cytokines propagate the inflammatory response, promote excitotoxicity, and oxidative injury giving rise to the neurotoxicity (10, 12). On the other hand, neuroinflammation attenuates central nervous system (CNS) damage through angiogenic, neurotrophic, and other mechanisms (13, 14). However, mTBI-induced changes of inflammatory cytokines and its association with cognitive consequences have not been investigated.

Cytokine profiles have been linked to adverse events and poor global outcomes after moderate to severe TBI (15, 16). For example, in children with TBI, IL-1β level in the first 24 h was negatively correlated 6 months later with Glasgow Outcome Scale (GOS) score (17). Previous studies investigating the functional significance of cytokine levels after mTBI have largely been cross-sectional in design (18), and have provided limited information. Indeed, little is known about the effects of mTBI-induced changes in inflammatory cytokine levels on the recovery of cognitive function. As such, there is a need for a systematic, prospective longitudinal study that can address this point.

To this end, the present study investigated the changes in serum levels of cytokines shortly after mTBI (<7 days) and 3 months later and the association between cytokine profiles and cognitive deficits. We further calculated a cytokine load score (CLS) as a measure of overall inflammatory burden to assess the predictive value of inflammation for cognitive outcome following mTBI (19, 20).

MATERIALS AND METHODS

Participants

Consecutive patients who underwent non-contrast head computed tomography after acute head trauma at local emergency department between August 2016 and June 2017 constituted Cohort 1; these patients were followed-up at 1 and 3 months post-injury so that their recovery could be monitored. Cohort 2 comprised patients recruited from August 2017 to December 2017; there were no follow-up of this group.

Inclusion criteria for mTBI patients were based on those outlined by the World Health Organization Collaborating Centre for Neurotrauma Task Force (21): (i) initial Glasgow Coma Scale score of 13–15; (ii) one or more of any following: loss of consciousness <30 min, post-traumatic amnesia <24 h and/or other transient neurological abnormalities such as focal symptoms and seizure; and (iii) diagnosed within 1 week of having experienced mTBI. Exclusion criteria were as follows: (i) pre-TBI, neurological or psychiatric illness diagnosed prior to TBI; (ii) drug or alcohol addiction; and (iii) mTBI occurring as a complication of other injuries (e.g., systemic, facial injuries, or spinal cord injury) or other problems (e.g., psychological trauma, language barrier, or coexisting medical conditions), or caused by penetrating craniocerebral injury.

Cohort 1 comprised 52 patients; blood samples were collected within 7 days post-injury (T1: 2.63 ± 1.23 days, all presented as Mean ± SD) and at 1-month (T2: 38.56 ± 9.33 days post-injury) and 3-months (T3:110.58 ± 21.13 days post-injury) follow-ups, reflecting the time course of recovery following mTBI (22). Neuropsychological tests were performed within 24 h of blood sampling. Cohort 2 comprised 43 mTBI patients within 7 days post-injury (T1: 3.16 ± 1.74 days).

In addition, 54 age-, gender-, and education level-matched healthy control subjects were recruited according to same set of exclusion criteria as those applied to mTBI patients. All participants provided written, informed consent for their participation and the study was approved by a local institutional review board and carried out in accordance with the tenets of the Declaration of Helsinki.

Serum Biomarker Detection

Serum samples were collected in the morning at 07:00–08:00 h and centrifuged, and aliquots of supernatant were stored at −80°C until analysis. Serum cytokine levels (pg/ml) were measured using reagents on a Luminex multiplex bead system (Luminex Austin, TX, USA). A fluorescence detection laser optic system was used to simultaneously detect binding of each individual protein onto microspheres, thereby allowing analysis of several analytes in a single sample. Intra- and inter-assay coefficients of variation for Luminex quantification were <20 and 25%, respectively. Samples with levels that were undetectable by the assay were set to 0.01 pg/ml. The criteria for the choice of cytokines were mainly based on whether it's associated with TBI or clinical symptoms such as PCS and cognitive function in previous studies (7, 16, 23). According to their effects on inflammation, cytokines we selected can be grouped as (i) the archetypal pro-inflammatory cytokines: IL-1β, IL-6, and IL-12, and the anti-inflammatory cytokines IL-4, IL-10; (ii) chemokine (C-C motif) ligand 2 or monocyte chemoattractant protein-1(CCL2 or MCP-1) and member of the CXC chemokine family (CXCL8) IL-8; (iii) interferon-γ (IFN-γ); and (iv) tumor necrosis factor α (TNF-α).

Neuropsychological Tests

Cognitive testing were performed within 24 h of blood sample collection and included (i) Trail-Making Test Part A (TMA) and Digit Symbol Coding score (DSC) from the Wechsler Adult Intelligence Scale III (WAIS-III) to measure cognitive information processing speed (24); (ii) Forward Digit Span and Backward Digit Span from the WAIS-III to evaluate working memory (25); (iii) Verbal Fluency Test to assess verbal fluency including language ability, semantic memory and executive function (26). And post-concussive symptoms (PCS) were evaluated with the Rivermead Post-Concussion Symptom Questionnaire (RPQ) (27). The tests were administered in face-to-face interviews by two psychologists blinded to the nature of the study.

Statistical Analysis

Statistical analyses were performed using the SPSS v.21 (IBM Corp, Armonk, NY, USA) and Prism v.5 (GraphPad Inc., La Jolla, CA, USA). The independent two-sample *t*-test and Mann Whitney test were used to evaluate differences between mTBI and control groups in acute phase based on the assumption of data normality. The chi-square test was used to evaluate categorical variables. General linear model was used to compare cognitive performance between patients and controls after adjusting for age and education level. Statistical significance was defined by an unpaired, two-tailed $P < 0.05$, except for an adjusted $P < 0.0056$ (0.05/9) for comparisons of the nine cytokines with Bonferroni correction.

Due to the skewed distribution of data, Friedman test was used to examine changes in cytokines levels, CLS and neuropsychological scores as a function of recovery after mTBI at three time points. The Bonferroni *post-hoc* correction for multiple comparisons was applied, yielding an adjusted $P < 0.0167$ (0.05/3) for three time points in mTBI group; this was compared to the values in the control group with Mann-Whitney test with Bonferroni correction, yielding an adjusted $P < 0.0167$ (0.05/3). In addition, percentage changes of cytokine levels, CLS and neuropsychological scores from acute phase over during of follow ups were determined by (T2-T1)/T1 and (T3-T1)/T1.

Inflammation-related cytokines showing significant inter-group differences in acute phase were used to calculate the CLS (28). Cytokine levels in the control group were divided into deciles; patients and control subjects with values higher than the 90th decile or lower than 10th decile were assigned values of "10" and "1," respectively. The values for all cytokines were then summed to obtain CLS. The capacity of cytokines and CLS to distinguish patients from controls was assessed by generating a receiver operating characteristic (ROC) curve and calculating area under the curve (AUC).

Stepwise multivariate linear regression analysis was carried out to determine the association between elevated cytokine levels and CLS in acute phase and neuropsychological scores at different time points following mTBI. The cytokines levels or CLS, along with age, gender and number of years of education were introduced stepwise into model as independent variables, with neuropsychological test scores as dependent variables. The criterion for entry or removal of a variable was *F*-value of 0.05. The multiple linear regression analysis was based on the following assumptions: the relationship between independent and dependent variables was linear; errors between independent and dependent values were normally distributed; and no multicollinearity was found. Given the multiple cognitive tests at different time points and the use of various non-independent measures in the tests, we calculated separate alpha thresholds in each of the main analyses to decrease the probability of type II errors (29). The significance threshold of associations between PCS and cytokine levels or CLS was set at $P < 0.0167$ (three time points = three tests). The significance threshold of associations between cognitive test score and cytokine levels or CLS was set at $P < 0.003$ (three time points × five cognitive tests = 15 tests).

RESULTS

Characteristics of the Study Population

There were no significant differences between mTBI Cohorts 1 and 2 in terms of clinical characteristics (**Supplementary Table S1**). Data from Cohort 2 ($n = 43$) and Cohort 1 ($n = 52$) were therefore combined, yielding a pooled sample of 95 patients in acute phase. The clinical characteristics of 95 patients are presented in **Table 1**. There were no differences in age, gender, and education level between mTBI patients and controls subjects. The most common cause of injuries was acceleration/deceleration caused by accident (60/95, 63.2%), followed by assaults (21/95, 22.1%), ground-level fall (7/95, 7.4%), fall from height (6/95, 6.3%), and direct impact blow to head (1/95, 1.1%).

Neuropsychological Measures

Patients reported significant discomfort in post-concussive symptoms (PCS) on scale of the RPQ ($P < 0.001$). There was worse performance in information processing speed assessed by the Digit Symbol Coding task (DSC) ($P < 0.001$), Trail-Making Test Part A (TMA) ($P = 0.012$) relative to controls after adjusting for age and education level. Besides, patients in Cohort 2 showed worse performance in Language Fluency test compared with control subjects ($P = 0.018$) (**Table 1**).

A longitudinal analysis of Cohort 1 showed that the main effects of time were significant for PCS ($\chi^2 = 14.241$, $P = 0.001$), TMA scores ($\chi^2 = 19.240$, $P < 0.001$) and DSC scores ($\chi^2 = 38.821$, $P < 0.001$), suggesting that PCS and cognitive function improved with time after mTBI. A *post-hoc* analysis revealed significant recovery in the TMA and DSC at T2 (TMA, $P = 0.013$; DSC, $P = 0.006$) and T3 (TMA, $P < 0.001$; DSC, $P < 0.001$) compared with T1, with the scores reaching control level at T2 (both $P > 0.0167$). Median of percentage changes (interquartile range) of TMA and DCS from T2 to T1 were −17.35% (43%) and 12.55% (42%), and from T3 to T1 were −22.85% (42%) and 19.68% (59%), respectively. However, although PCS was improved at T2 ($P = 0.002$) and T3 ($P = 0.001$) compared with T1, they were more severe than in controls at T3 ($P < 0.001$); median of percentage changes of PCS in T2 and T3 were −48.08% (71%) and −47.22% (100%) (**Supplementary Figures S1, S2**).

TABLE 1 | Summary of demographic and clinical information for mTBI patients and normal control subjects.

Demographic[a]	mTBI ($n = 95$)	Controls ($n = 54$)	*P*-value
Age in years	35.93 ± 13.69 (33.14–38.71)	35.74 ± 11.51 (32.60–38.88)	0.987
Gender	55M:40F	29M:25F	0.731
Education in years	8.51 ± 3.75 (7.74–9.27)	9.43 ± 4.14 (8.30–10.55)	0.103
Neuropsychological tests[a]			
Trail Making A	62.32 ± 45.93 (52.96–71.67)	41.70 ± 23.55 (35.27–48.13)	**0.012**
Digit symbol coding	34.91 ± 15.85 (31.68–38.13)	46.93 ± 16.88 (42.32–51.53)	**<0.001**
Digit span-forward	7.84 ± 1.53 (7.53–8.15)	8.33 ± 1.67 (7.87–8.79)	0.653
Digit span-backward	3.79 ± 1.34 (3.51–4.06)	4.51 ± 1.90 (4.00–5.04)	0.233
Language fluency	16.14 ± 5.16 (15.09–17.19)	18.93 ± 6.51 (17.15–20.70)	0.109
Symptoms severity[a]			
PCS	10.27 ± 7.26 (8.79–11.75)	2.33 ± 2.83 (1.56–3.11)	**<0.001**
mTBI severity *n* (%)			
Loss of conscious	86 (90.5%)	NA	
Post-traumatic amnesia	9 (9.5%)	NA	
GCS = 15	95 (100%)	NA	
GCS = 13, 14	0 (0%)	NA	
Causes for mTBI *n* (%)			
Acceleration/deceleration	60 (63.2%)	NA	
Ground level fall	7 (7.4%)	NA	
Fall from height	6 (6.3%)	NA	
Assaults	21 (22.1%)	NA	
Direct impact blow to head	1 (1.1%)	NA	

[a]*Continuous variables are expressed as mean ± SD (90% confidence intervals) and categorical variables are expressed as a frequency and percentage. Results of neuropsychological tests are presented as raw scores. Statistically significant P values are shown in bold. GCS, Glasgow Coma Scale; mTBI, mild traumatic brain injury; NA, non-available; PCS, Post-concussive symptoms.*

Serum Cytokine Levels and Determination of CLS

Serum levels of CCL2, IL-1β, and IL-6 in acute phase were higher in mTBI patients than in controls (all $P < 0.001$) after Bonferroni correction with an adjusted $P < 0.0056$ (0.05/9 for nine cytokines; **Figure 1** and **Supplementary Table S2**; given that the values were not normally distributed, thus presented as median and interquartile ranges). AUCs calculated from ROC curves of each cytokine ranged from 0.070 to 0.078, which distinguished mTBI patients from control subjects (**Supplementary Figure S3**).

The cytokines that were significantly elevated in acute phase of mTBI (IL-1β, IL-6, and CCL2) were used to calculate CLS, which was higher in mTBI patients than in controls ($P < 0.001$) (**Figure 2A**). AUC calculated from ROC curve of CLS was able to discriminate between the mTBI and control groups (AUC = 0.83, 95% confidence interval = 0.76–0.89, $P < 0.001$; **Figure 2B**).

A longitudinal analysis of Cohort 1 showed that the main effects of time were significant for CCL2 ($\chi^2 = 25.239$, $P < 0.001$), IL-1β ($\chi^2 = 19.408$, $P < 0.001$), IL-6 ($\chi^2 = 9.169$, $P = 0.010$), and CLS ($\chi^2 = 7.320$, $P = 0.026$; **Figures 2C**, **3**). In *post-hoc* analysis, CCL2 level was higher at T2 ($P < 0.001$) and T3 ($P < 0.001$) relative to T1, and median of percentage changes (interquartile range) were 35.04% (62%) and 29.37% (69%) in T2 and T3 (**Figure 4**); IL-1β showed no significant change between T1 and T2 ($P = 0.011$) but was decreased at T3 compared with T2 ($P < 0.001$); and IL-6 level was lower at T3 compared with T1 ($P = 0.003$). Median of percentage changes for IL-1β and IL-6 were 8.17% (29%) and −13.78% (67%) in T2 and −8.07% (33%) and −23.07(50%) in T3 (**Figure 4**). CLS showed no significant differences among mTBI groups and its median of percentage changes were 6.07% (37%) and −2% (29%) in T1 and T2 (**Figure 2D**). Relative to control group, only IL-1β showed no difference at T3 ($P = 0.34$).

Relationship Between Cytokine Levels and Neuropsychological Test Scores

Stepwise multiple linear regression was performed; only one model including cytokines survived the multiple testing correction. In pooled analysis for acute phase ($n = 95$), a higher CCL2 level was associated with greater severity of PCS [CCL2, standardized β = 0.465, $P < 0.001$; overall model: $F_{(1, 93)} = 24.22$, $P < 0.001$, adjusted $R^2 = 0.198$; **Figure 5**]. Elevated IL-1β tended to be associated with poorer working memory as determined by Digit Span backward test [IL-1β, standardized β = −0.187, $P = 0.047$; overall model: $F_{(2, 92)} = 12.442$, $P < 0.001$, adjusted $R^2 = 0.196$], although this failed to pass correction.

In the follow up for Cohort 1 ($n = 52$), elevated CCL2 at T1 predicted a tendency for higher Digital Symbol coding test score at T3 [CCL2, standardized β = 0.214, $P = 0.009$; overall model: $F_{3, 48} = 43.509$, $P < 0.001$, adjusted $R^2 = 0.714$]; however, the significance of coefficient of independent variables failed to pass the correction.

To determine whether cytokine levels influence the recovery of PCS and cognitive measures from acute to chronic phase of mTBI, we calculated the changes in these scores by subtracting T1 from T3 in Cohort 1 ($n = 52$). A change in working memory as evaluated by Digit Span backward test tended to be associated with IL-1β level in acute phase [IL-1β, standardized β = 0.336, $P = 0.015$; overall model: $F_{1, 50} = 6.361$, $P = 0.015$, adjusted $R^2 = 0.095$], but this also failed to pass the correction.

DISCUSSION

In this study, serum cytokine levels were increased after mTBI and persisted from acute to chronic phase. Higher cytokine levels were associated with greater severity of PCS and worse working memory acutely. However, cytokine levels in acute phase showed a positive association with information processing speed at 3 months post-injury. This is the first study to investigate inflammatory cytokine profiles in mTBI patients and their

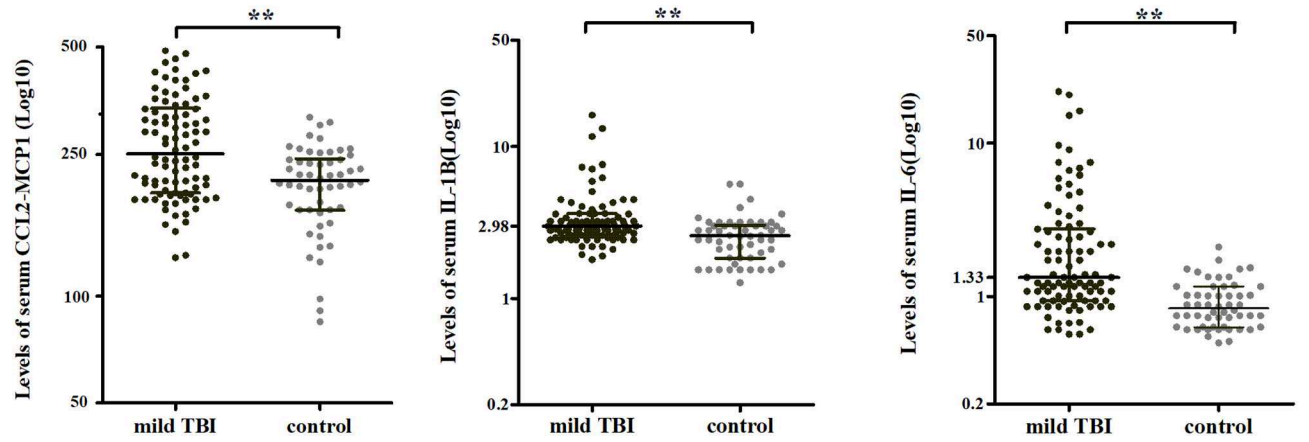

FIGURE 1 | Serum cytokine levels in mTBI patients and controls in acute phase. Serum levels of CCL2, IL-1β, and IL-6 were higher in mTBI patients (black, $n = 95$) than in controls (gray, $n = 54$). Horizontal bars represent the medians and interquartile range in pg/ml. $^{**}P < 0.001$.

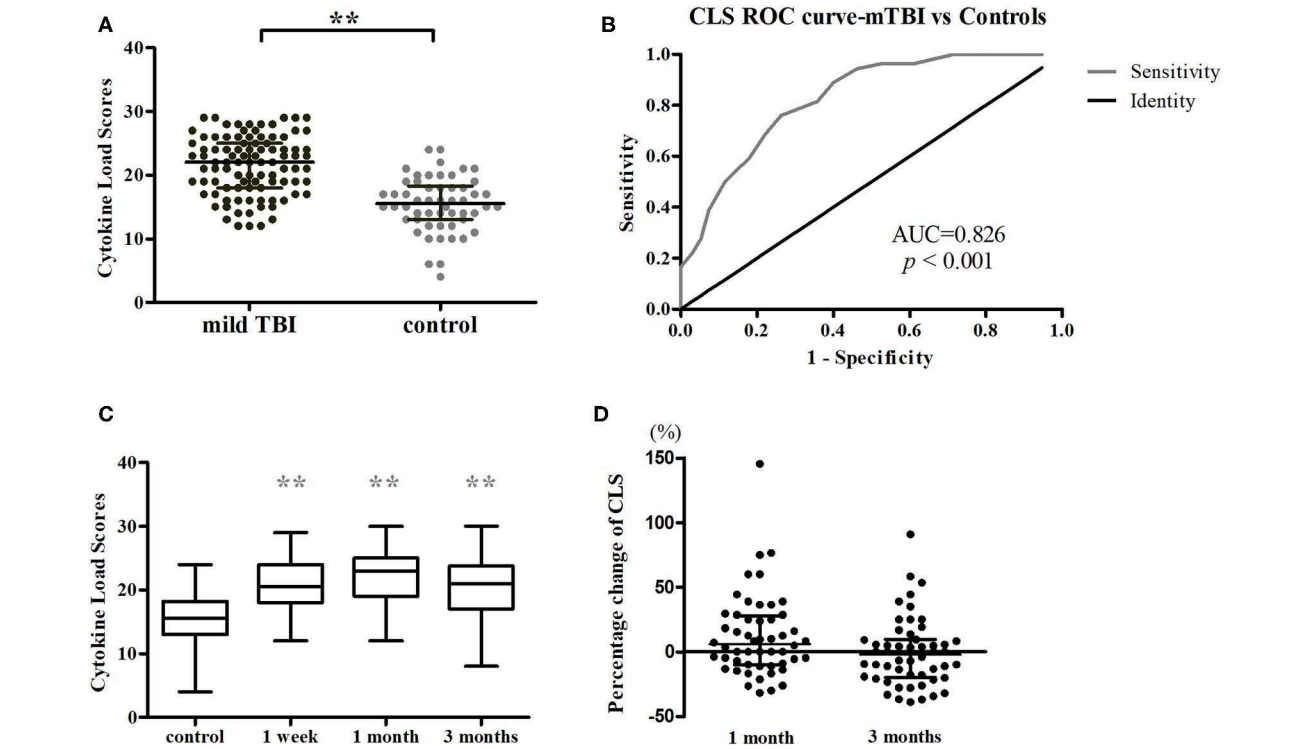

FIGURE 2 | Analysis of CLSs. **(A)** CLS was higher for mTBI patients ($n = 95$, black) than control subjects ($n = 54$, gray) in acute phase. $^{**}P < 0.001$. **(B)** CLS ROC curve. **(C)** Longitudinal changes in CLS after injury in Cohort 1 ($n = 52$ for all three time points). Horizontal bars represent the medians and interquartile range in pg/ml. Statistically significant differences between patients and controls at each time points and between different time points within patients are indicated by gray asterisks and black asterisks, respectively. $^{**}P < 0.001$. **(D)** Percentage changes of CLS from acute phase to follow-ups. Boxplots represent medians and interquartile ($n = 52$ for both time points).

relationship to cognitive dysfunction, which is a major factor contributing to poor prognosis in mTBI.

The inflammatory response to TBI is triggered by brain tissue damage and involves the activation of resident microglia, astrocytes, and peripheral inflammation (15, 16). Cytokines levels are elevated in acute phase after mTBI, but little is known about the changes in chronic phase (19, 30). In the present longitudinal study, we found that the levels of inflammation-related cytokines

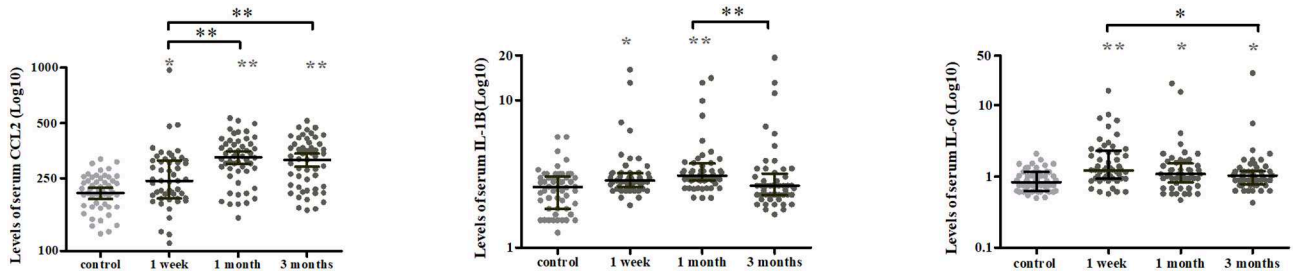

FIGURE 3 | Temporal profiles of serum cytokine levels over a 3-months period in mTBI patients. Scatter plots showing a higher CCL2 at 1 and 3 months while lower IL-1β and IL-6 levels at 3 months post-injury in mTBI patients. Horizontal bars represent the median and interquartile range in pg/ml ($n = 52$ for all three time points). Statistically significant differences between patients and controls at each time points and between different time points within patients are indicated by gray asterisks and black asterisks, respectively. *$P < 0.005$ and **$P < 0.001$.

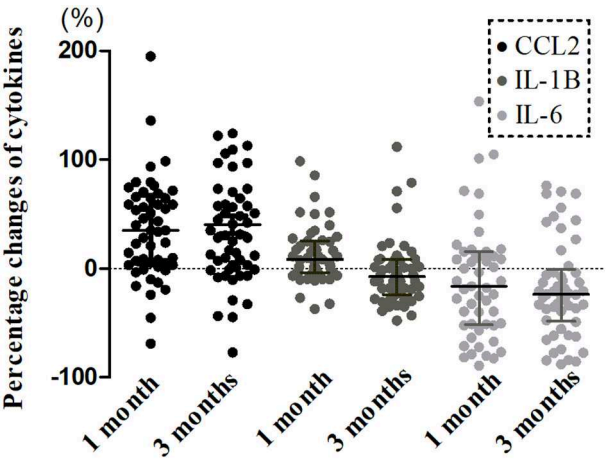

FIGURE 4 | Percentage changes from acute phase to follow-ups in levels of serum cytokines in mTBI patients. Boxplots represent medians and interquartile ($n = 52$ for both time points).

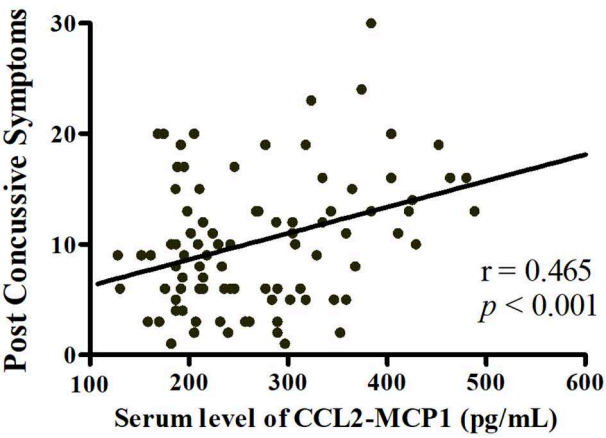

FIGURE 5 | Linear regression analysis of the relationship between neuropsychological test performance and serum cytokine levels. Higher serum CCL2 level was associated with greater severity of post-concussive symptoms in acute phase post-injury ($n = 95$).

were elevated at 3 months post-injury, suggesting that low-grade systemic inflammation persists in mTBI patients, as suggested by a study of C-reactive protein (CRP) levels (31). Animal and clinical studies based on neuropathological and position emission tomography observation have reported that microglial activation can continue for more than 1 year after TBI (11, 32, 33). Thus, mTBI-associated neuroinflammation in the CNS and at peripheral sites is long-lasting.

PCS were evaluated with RPQ and included physical, emotional, behavioral, and cognitive symptoms (such as headache, anxiety, fatigue, irritability, memory, and concentration problems, etc.) (27). In our study, the level of the inflammatory cytokine CCL2 was positively associated with PCS, which is consistent with previous studies (23, 31, 34). For instance, elevated levels of CRP and S-100β (a glial cell protein) in acute phase were associated with increased risk of persistent PCS (31, 34). Systemic inflammation can trigger neuroinflammation through circumventricular organs, vagal afferents, or the brain endothelium (35), undermining the microstructural integrity of white matter (36, 37), disrupting microglia function in synaptic plasticity (38) and reducing cognitive functioning. Stimuli causing neuroinflammation can directly injure the trigeminal afferent nerves or the leptomeningeal or cerebrovascular structures that they innervated, leading to post-traumatic headaches (39). Peripheral inflammation activates the hypothalamic-pituitary-adrenal (HPA) axis (40), which resulting in chronic stress-associated anxiety and depression (41). In general, systemic inflammation contributes to PCS. A new term post-inflammatory brain syndromes (PIBS) has been proposed to encompass the contribution of systemic inflammation to the development of PCS-like symptoms, even in patients without head injury (23).

In the current study, IL-1β level tended to be negatively associated with working memory in acute phase and positively associated with changes in working memory at 3 months post-injury. The former can be ascribed to the role of IL-1β in

mTBI-related cognitive impairment (42), whereas the latter could be due to a lower cognitive function at baseline, which would provide room for change compared with a normal level of inflammation that changes little over time.

Diffuse axonal injury caused by shear stress and tissue deformation often occurs in mTBI (43). Information processing speed depends on the integrity of the myelin sheath surrounding neuronal axonal fibers (44). During recovery from mTBI, secondary neuroinflammation contributes to white matter damage (45) and affects the speed of information processing. In our study, CCL2 level tended to be positively associated with information processing speed. Within hours of injury, astrocytes produce CCL2, the level of which is correlated with the number of recruited monocytes (46). The recruitment of peripheral monocytes to the meninges has been shown to exert beneficial effects on post-mTBI recovery by promoting meningeal remodeling and vascular repair (47).

In this study, we developed the CLS to reflect overall cytokine burden, which was more useful as a biomarker for mTBI than measurement of a single cytokine. It has been demonstrated that machine learning or principal component analysis of cytokine levels is more accurate for the diagnosis of mTBI and better predicts patient outcome than our approach (30, 48); therefore, additional studies are needed to confirm the diagnostic and predictive value of the pattern of immune response in TBI. While cytokine levels were found to be linked to the manifestation of neuropsychological symptoms, these associations were marginally significant and failed to pass the multiple testing correction. One reason for this is that multiple factors influence neuropsychological symptoms including environmental factors and psychological state (49, 50) as well as genetic factors and innate biological variability (51, 52). In addition, cytokines interact with other factors to activate complex downstream signaling networks (53). Future studies should focus on patterns of cytokine activity or expression in order to elucidate their network-level activity and function.

There were some limitations to this study. Firstly, we observed marked changes in serum cytokine levels during acute phase; a time course of 1 week may not have been sufficient to observe the actual dynamics of cytokines profiles. Secondly, serum cytokine levels do not directly reflect the CNS environment. Cerebrospinal fluid (CSF) is considered to provide data that are more reliable in this regard, but the collection of CSF samples involves lumbar puncture, which is poorly tolerated by patients. Finally, a longer follow-up time is needed to observe the full range of neuropsychological outcomes caused by inflammation-related cytokines in mTBI, especially given that macrophages and microglia remain active in the CNS for months or years post-injury and can adopt aberrant functions.

CONCLUSIONS

The results of our study indicate that persistent, low-grade systemic inflammation exists in mTBI patients. Higher cytokines levels were associated with a greater severity of PCS and worse cognitive function in acute phase. In addition, elevated levels of specific cytokines in acute phase were positively associated with cognitive outcome in the chronic phase. Our findings demonstrate that serum cytokine measurements provide important information on post-mTBI outcome, additional studies are needed to clarify the pathological basis of the relationship between inflammation and neuropsychological symptoms of mTBI through combining serum cytokine measurements and neuroimaging approaches.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The research procedures were approved by the Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University. All subjects provided written, informed consent prior to their participation in the study in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

YS contributed to the design of the Luminex experiment, analyzed data, drafted, and revised the manuscript. XN, ZW, SW, SG, and CS prepared the experimental materials and analyzed data. BY, GB, and DZ recruited the subjects and collected serum samples. FZ, LB, and MZ contributed to study conception and design and revised the article. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.01120/full#supplementary-material

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Methylene Blue Reduces Neuronal Apoptosis and Improves Blood-Brain Barrier Integrity After Traumatic Brain Injury

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Objective: To investigate whether methylene blue (MB) treatment can reverse neuronal mitochondrial dysfunction caused by oxygen glucose deprivation/reoxygenation (OGD) injury and then investigate whether MB treatment can reduce neuronal apoptosis and improve blood-brain barrier (BBB) integrity in traumatic brain injury (TBI) animals.

Methods: Reactive oxygen species (ROS), mitochondrial membrane potential (MMP), and adenosine triphosphate (ATP) were used to evaluate mitochondrial function. The terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) assay was used to assess neuronal apoptosis *in vitro*. TUNEL and immunofluorescence staining for neuronal nuclei (NeuN) were combined to assess neuronal apoptosis *in vivo*. An Evans blue (EB) permeability assay and brain water content (BWC) were used to measure BBB permeability *in vivo*. The Morris water maze (MWM), rotarod test, and modified Neurological Severity Score (mNSS) test were employed to assess the prognosis of TBI mice.

Results: MB treatment significantly reversed neuronal mitochondrial dysfunction caused by OGD injury. Both *in vitro* and *in vivo*, MB treatment reduced neuronal apoptosis and improved BBB integrity. In TBI animals, treatment with MB not only improved cognitive and motor function caused by TBI but also significantly improved overall neurological function.

Conclusions: Our findings suggest that MB is a potential candidate for the treatment of TBI. Future research should focus on other therapeutic effects and mechanisms of MB in secondary brain injury.

Keywords: adenosine triphosphate, apoptosis, blood-brain barrier, methylene blue, reactive oxygen species, traumatic brain injury

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INTRODUCTION

Traumatic brain injury (TBI) is the most common cause of mortality and disability among working-age adults and young individuals worldwide (1). In the United States, ∼2 million people suffer a TBI each year, and TBI accounts for nearly one-third of all trauma-related mortality (1, 2). TBI damages brain tissue through two pathological processes, primary and secondary injury. Primary injury is

characterized by immediate bleeding and loss of brain tissue when a blunt or sharp object impacts the head. Secondary injury involves complicated cellular and biochemical cascade reactions, including oxidative stress, excitotoxicity, neuroinflammation, free radical-induced injury, and calcium-mediated damage, which lead to blood-brain barrier (BBB) damage, elevated intracranial pressure, cerebral hypoxia, brain edema, and neuronal apoptosis (3–8). Mitochondrial dysfunction has been demonstrated to be a key participant in the pathological processes of secondary brain injury (9, 10).

Methylene blue (MB) is an FDA-approved drug used to treat cyanide poisoning, carbon monoxide poisoning, and methemoglobinemia (11). Previous studies have demonstrated that MB can improve mitochondrial function (12). Under pathological conditions, MB acts as an alternative electron carrier that bypasses complex I/III blockage and efficiently transfers electrons from NADH to cytochrome c (cyt c). This process reduces electron leakage, enhances adenosine triphosphate (ATP) production, and decreases the overproduction of reactive oxygen species (ROS) (13). In recent years, MB has been shown to attenuate pathological and neurobehavioral impairments in animal models of Alzheimer's disease (AD) (14, 15), Parkinson's disease (PD) (16), ischemic stroke (17, 18), and TBI (19–21). After TBI, MB treatment can attenuate neuroinflammation, reduce lesion volume, and improve neurological damage (19–21).

Since MB treatment can reduce the release of ROS and increase the production of ATP, it may have the potential to reduce neuronal apoptosis and improve BBB integrity. However, these effects of MB on TBI have not been investigated. In the present study, we first investigated whether MB treatment can reverse neuronal mitochondrial dysfunction and then investigated whether MB treatment can reduce neuronal apoptosis and improve BBB integrity after TBI.

MATERIALS AND METHODS

Normal Cells Culture and Oxygen Glucose Deprivation/Reoxygenation (ODG) Model

PC-12 cells (ATCC, Manassas, VA, USA) and Bend3 cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle medium (DMEM) (BioInd, Kibbutz Beit Haemek, Israel) supplemented with 100 units/ml streptomycin/penicillin (HyClone, Utah, Logan City, USA) and 10% fetal bovine serum (FBS) (BioInd, Kibbutz Beit Haemek, Israel). Normal cultured cells were maintained in a 5% CO_2 atmosphere at 37°C. For the OGD model, cells were incubated in glucose-free DMEM and placed in an anaerobic chamber with 5% O_2, 5% CO_2, and 80% N_2 at 37°C for 4 h. A BD Disposable Anaerobic Indicator was used to measure the oxygen level of the anaerobic chamber. After OGD incubation, the glucose-free DMEM was replaced with normal culture medium, and the cells were maintained under normal culture conditions.

Animals

All animal experimental procedures in this study were approved by the Yijishan hospital and Tianjin Medical University Animal Ethics. Male, weighing 1,822 g (6–8 weeks old), C57BL/6 mice were bought from Experimental Animal Laboratories of the Academy of Military Medical Sciences (Beijing, China). The environment of the animal room was set to 12 h light-dark cycle with a temperature controlled at 20 ± 2°C and humidity controlled at 55 ± 5%. Animals were housed with free access to food and water before experimentation.

MB Treatment

OGD cells were divided into two groups, the untreated and MB (Jichuan, Taixing, China) treatment groups. In the MB treatment group, the culture medium was treated with 4.5 μM 1% MB (20) at the same time OGD began and was replaced with normal culture medium at the end of OGD. In animals, MB was injected intraperitoneally 1 h after TBI (1 mg/kg), 6 h after TBI (0.5 mg/kg), and at a dosage of 1 mg/kg daily for the next 3 days. The control groups received intraperitoneal injections of the same volume of saline (22).

Detection of ROS Production

ROS levels were detected using an ROS Detection Kit (BestBio, ShangHai, China). After normal or OGD incubation, the fluorescent probe DCFH-DA (1:2,500) was added to the PC-12 cells, and the cells were incubated for 30 min. The cells were then resuspended after washing and centrifugation, transferred to a flow tube, and analyzed using flow cytometry.

Mitochondrial Membrane Potential (MMP) Measurement

The MMP of the neurons was measured by a JC-1 Kit (Solarbio, Beijing, China). After normal or OGD incubation, the prepared JC-1 staining working fluid was added to the cells, and the cells were incubated for 20 min at 37°C and then washed with JC-1 staining buffer. Then 2 ml of DMEM was added to each well, and the cells were observed under a fluorescence microscope. The ratio of green fluorescence to red fluorescence was used to represent changes in the MMP.

ATP Measurement

ATP was measured using an ATP Assay Kit (Beyotime, Shanghai, China). A total of 200 μl of lysate was added to each well of a six-well plate to lyse the cells, and then the cells were centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was transferred as a specimen for testing. Then 100 μl of ATP test solution was mixed with 10 μl of test specimen or standard specimen, and the optical density (OD) values of the mixture were measured with a spectrophotometer. The ATP concentration was calculated according to the standard curve.

Establishment of the BBB Model and Permeability Detection

The BBB model was established using a Transwell Kit (diameter 24 mm, pore size 0.4 μm, Corning, NY, USA) according to the manufacturer's instructions. Bend3 cells (1×10^6/ml, 250 μl) were seeded in a travel chamber, and the chamber was transferred to a 12-well plate containing 500 μl of DMEM per well. After incubation for 48 h, the DMEM was renewed, and the cells were incubated for 4 h under normal oxygen or hypoxic conditions.

FITC-Dextran (Sigma-Aldrich, St Louis, MO, USA) (250 μl) was added to each chamber, and the cells were incubated for 90 min. Then 100 μl of the solution in the well below the chamber was transferred to a 96-well plate, and the OD (535 nm) values were measured by a spectrophotometer.

Terminal Deoxynucleotidyl Transferase-dUTP Nick End Labeling (TUNEL) Assay

For cells, the PC-12 cells were plated on glass coverslips after normal or OGD incubation, fixed with 4% paraformaldehyde for 1 h and permeabilized with 0.3% Triton X-100 for 2 min. A total of 50 μl of TUNEL mixture (Roche, Nutley, NJ, USA) was added to each sample, and the cells were incubated for 60 min at 37°C and then incubated with 4',6-diamidino-2-phenylindole (DAPI). For tissue samples, TUNEL and immunofluorescence staining of neuronal nuclei (NeuN) were combined to assess neuronal apoptosis. Frozen sections were prepared 3 days post-TBI and were incubated with an anti-NeuN antibody (1:500, Abcam, Cambridge, MA, USA) at 4°C overnight. After 1 h incubation with Alexa Fluor-conjugated anti-rabbit or anti-mouse IgG (1:500, Thermo Fisher Scientific, Waltham, MA, USA), 50 μl of TUNEL mixture was added, and the sections were incubated for 1 h at 37°C and then incubated with DAPI. A fluorescence microscope was used to determine the number of apoptotic neuronal cells around the traumatic foci. The neuronal apoptosis ratio was recorded for statistical analysis.

TBI Model

A fluid percussion injury device (Model 01-B, New Sun, Health Products, Cedar Bluff, USA) was used to establish a TBI model. Mice were anesthetized with 10% chloral hydrate by intraperitoneal injection (3 ml/kg) and then placed in a stereotaxic frame. After the scalp was incised sagittally, a 3.5-mm diameter opening was drilled in the right cranium 2.0 mm lateral to the sagittal suture between bregma and lambda. For the sham groups, the surgical procedure was completed. For the experimental groups, a Luer lock connector was placed in the skull opening and cemented in place with cranioplastic cement. The Luer lock was filled with 0.9% normal saline and connected to the fluid percussion device. TBI was induced using controlled fluid percussion as described previously. The incision was sutured immediately, and the animal was placed on a heating pad for recovery from anesthesia.

Evans Blue (EB) Permeability Assay

For analysis of BBB permeability, mice were injected with 2% EB (3 ml/kg) (Sigma-Aldrich, Tokyo, Japan) *via* the tail vein 72 h after TBI (23). After an hour, the mice were anesthetized with 10% chloral hydrate and then perfused with phosphate buffer saline (PBS) to purge the intravascular EB dye. After decapitation, the ipsilateral hemibrains of the TBI and sham groups were weighed and homogenized in 0.1 g/ml *N,N*-dimethylformamide (Sigma-Aldrich, Tokyo, Japan). Following incubation for 48 h in a 37°C water bath, the hemispheres were centrifuged at 3,000 rpm for 30 min. The supernatants were collected, and the EB absorbance was measured using a spectrophotometer. The concentration was determined from the OD (610 nm) values according to the standard curve, and then the EB content in the brain tissue was calculated.

Brain Water Content (BWC)

The severity of cerebral edema was evaluated by BWC using the wet weight-dry weight method. Mice were sacrificed 72 h post-TBI, and after anesthesia and decapitation, the brains were removed immediately and divided into the ipsilateral hemisphere, contralateral hemisphere, cerebellum, and brainstem. The fresh tissue samples were weighed immediately to obtain the wet weight and then dried at 100°C for 24 h to obtain the dry weight. The percentage of water content was calculated as follows: brain water content = [(wet weight – dry weight)/wet weight] × 100%.

Western Blot Analysis

Protein was extracted from injured brain tissues using RIPA buffer, and the concentration was measured by a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Approximately 10 μg of protein per lane was separated using an SDS-PAGE system and transferred to PVDF membranes. The membranes were then blocked with 5% BSA for 1 h at room temperature and incubated with primary antibodies (Caspase 3, 1:1,000, Cell Signaling Technology, Inc., MA, USA; ZO-1, 1:1,000, Cambridge, MA, USA) in 3% BSA at 4°C overnight. After incubation with secondary antibodies (goat anti-mouse or anti-rabbit IgG, 1:5,000, Zsbio, Beijing, China) at room temperature for 1 h, the blots were developed by western lightning chemiluminescence reagents and detected using a Millipore ECL Western Blotting Detection System (Millipore, Billerica, MA, USA).

Morris Water Maze (MWM)

The MWM test was used to assess cognitive deficits in mice. A circular container (50 cm deep, 150 cm wide) was filled with water at a suitable temperature (22 ± 2°C) and white dye, and a hidden platform was fixed in the center of the container and submerged 1 cm below the water surface. The container was located in a 2 × 2 m room with cues (a square, star, triangle, and circle) on the walls. The data were captured automatically using a video tracking system (Ethovision 3.0; Noldus Information Technology, Wageningen, Netherlands) connected to a computer. All mice were trained for 5 consecutive days after 14 days post-TBI. In spatial learning training, the mice were placed into the container in a random quadrant and allowed to swim in the container until they found the platform. If a mouse failed to find the platform within 60 s, the investigator picked it up and placed it on the platform for 30 s. The platform was removed to evaluate memory retention. The number of crosses into the platform quadrant and the percentage of time spent in the platform quadrant in 60-s intervals were measured.

Rotarod Test

An accelerating rotarod (RWD Life Science, Shenzhen, China) was used to assess motor coordination and balance. The animals were trained daily for 2 days prior to TBI and were tested 24 h

after TBI. Speed gradually increased from 4 rpm to 20 rpm over a 5-min period, and the time that the mice stayed on the rotating cylinder was automatically recorded. The mice underwent three trials with an interval of more than 30 min, and the time spent on the cylinder was averaged over three trials (24).

The Modified Neurological Severity Score (mNSS)

mNSS measurements were used to evaluate posttraumatic neurological function according to a previous study (24). The test consists of motor (muscle status, abnormal movement), sensory (visual, tactile and proprioceptive), balance beam, and reflex tests. mNSS ranged from 0 (normal function) to 18 (maximal deficit). In the present study, mNSS were assessed on days 1, 3, 5, 9, and 14 days post-TBI. mNSS assessments were carried out by two observers who were blinded to the groups.

Statistical Analysis

All cellular experiments were repeated at least three times. All experiments were performed in a randomized and blinded manner. SPSS statistical software (version 22.0, IBM) was used for all statistical analyses in the present study. The results are presented as the mean ± SD (standard deviation) and were analyzed using a *t*-test between two groups. One-way ANOVA followed by multiple comparison by LSD test was used for comparisons between multiple groups. The protein band intensity for the Western blots and the fluorescence intensity for MMP measurement were determined using ImageJ software. A P-value < 0.05 was statistically significant.

RESULTS

MB Treatment Decreases ROS Production Caused by OGD Injury

After 4 h of OGD incubation, the production of ROS by neurons was significantly increased compared with that of the normal incubation group ($P = 0.004$). The combination of MB treatment and OGD significantly decreased the production of ROS compared with that induced by OGD incubation alone ($P = 0.044$) (**Figures 1A,B**). This result suggests that MB treatment can significantly reduce neuronal ROS production under OGD injury.

MB Treatment Stabilizes the Neuronal MMP After OGD Injury

As shown in **Figures 1C,D**, we used the ratio of green fluorescence signal intensity to red fluorescence signal intensity to represent the stability of the MMP. The stability of the neuronal MMP was significantly reduced in the OGD group compared with the normal incubation group ($P = 0.011$), while MB treatment significantly reversed the decline of MMP stability caused by OGD injury ($P = 0.033$).

MB Treatment Increases the Production of ATP in Injured Neurons

To determine whether MB treatment can increase the production of ATP in injured neurons, we examined the ATP concentration in the control group, OGD group, and OGD + MB group. As shown in **Figure 1E**, the ATP concentration was obviously lower in the OGD group than in the control group ($P = 0.011$), and MB treatment significantly decreased ATP consumption compared with that in the OGD group ($P = 0.039$). MB treatment can reduce ROS production, reverse the decline of MMP stability, and increase ATP consumption in neurons under OGD injury. These results demonstrate that MB treatment can reverse the mitochondrial dysfunction caused by OGD injury.

MB Treatment Decreases Neuronal Apoptosis Caused by OGD Injury

Because MB can reverse the mitochondrial dysfunction caused by OGD injury, we speculate that it can reduce neuronal apoptosis after OGD injury. The results showed that the proportion of apoptotic neurons in the OGD group was significantly increased compared with that in the normal cultured group ($P = 0.002$), but the proportion of apoptotic neurons in the OGD with MB treatment group was significantly decreased compared with that in the OGD group ($P = 0.008$) (**Figures 2A,B**). This result indicates that MB treatment can decrease neuronal apoptosis after OGD injury *in vitro*.

MB Treatment Can Improve the Integrity of the BBB *in vitro*

A BBB model was established to evaluate whether MB treatment can improve the integrity of the BBB *in vitro*. As shown in **Figure 2C**, the permeability of the BBB model was significantly increased in the OGD group compared with the control group ($P < 0.001$), and MB treatment significantly improved the integrity of the BBB ($P < 0.001$).

MB Treatment Decreases Neuronal Apoptosis Caused by TBI *in vivo*

Since MB treatment can improve mitochondrial function, reduce neuronal apoptosis, and improve BBB permeability after OGD injury *in vitro*, we hypothesized that the administration of MB after TBI can also reduce neuronal apoptosis, improve BBB permeability, and reduce brain edema *in vivo*. To confirm our speculation, neuronal apoptosis, BBB permeability, and brain water content in animal models were assessed. The timeline of the animal experiments is presented in **Figure 3**. As shown in **Figures 4A,B**, double staining for NeuN and TUNEL revealed that TUNEL-positive cells were mainly neurons. In the right cerebral cortex of the sham group and the sham + saline group, almost no neuronal apoptosis was observed. In the cortex around the lesion in the TBI group, neuronal apoptosis was significantly increased (vs. the sham + saline group, $P < 0.001$; vs. the sham + MB group, $P < 0.001$), whereas MB treatment promoted neuronal survival (vs. the TBI + saline group, $P < 0.001$). To further confirm that MB can reduce TBI-induced neuronal apoptosis. We examined caspase 3 expression between the groups. The expression of caspase 3 was significantly higher in the TBI + saline group than in the sham + saline group ($P = 0.003$) and sham + MB group ($P = 0.002$), whereas MB treatment significantly reduced caspase 3 expression after TBI ($P = 0.02$) (**Figure 4C**).

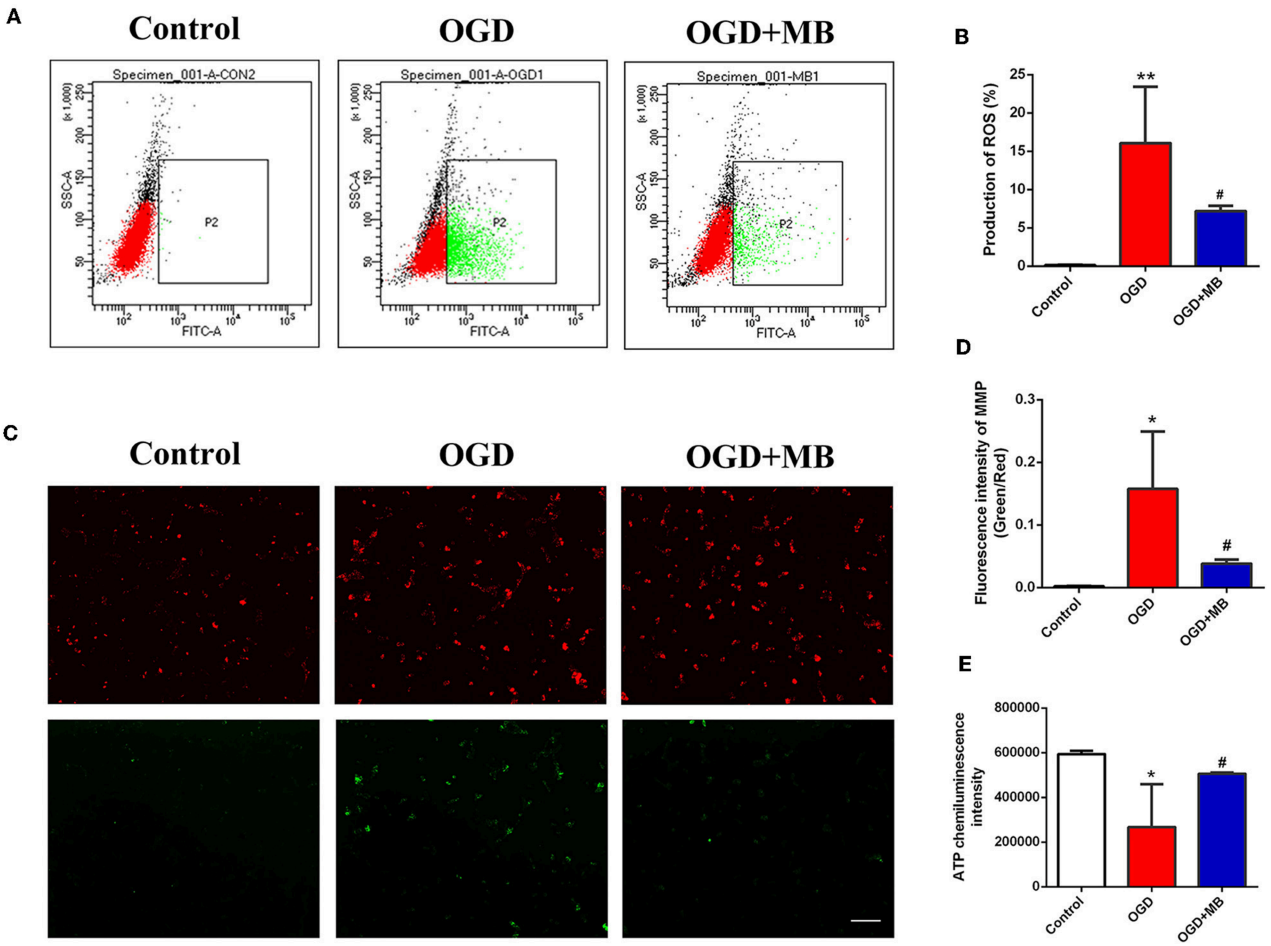

FIGURE 1 | Methylene blue (MB) treatment reversed neuronal mitochondrial dysfunction caused by oxygen glucose deprivation/reoxygenation (OGD) injury. **(A)** Green dot plots represent the level of ROS. **(B)** Quantitative analysis of the reactive oxygen species (ROS) production. $^{**}< 0.01$, vs. Control group, $^{\#} < 0.05$, vs. OGD group. **(C)** Green fluorescence/red fluorescence ratio represents the MMP stability. Scale bar = 200 μm. **(D)** Quantitative analysis of the MMP, $^{*}< 0.05$, vs. Control group, $^{\#} < 0.05$, vs. OGD group. **(E)** OD value represents the adenosine triphosphate (ATP) level. $^{*}< 0.05$, vs. Control group, $^{\#} < 0.05$, vs. OGD group.

MB Decreases EB Permeability and Reduces Brain Water Content After TBI

To determine whether MB treatment can also improve the decreased integrity of BBB caused by TBI *in vivo*, both EB permeability and brain water content were evaluated. Three days after TBI, the EB permeability in the TBI + saline group was significantly increased compared with that in the sham + saline group ($P = 0.013$) and the sham + MB group ($P = 0.011$). In the TBI + MB group, the EB permeability was significantly lower than that in the TBI + saline group ($P = 0.047$) (**Figures 5A,B**). The water content of brain tissue from the contralateral side and of the cerebellum and brainstem did not differ between the groups. The water content of brain tissue from the ipsilateral side was significantly higher in the TBI + saline group than that in the sham + saline group ($P = 0.048$), sham + MB group ($P = 0.013$), and TBI + MB group ($P = 0.041$) (**Figure 5C**). These results indicate that MB treatment can also decrease the BBB permeability caused by TBI. ROS increases the permeability of the BBB by downregulating the expression of the tight junction protein ZO-1 after TBI (4); therefore, ZO-1 expression in the different groups was detected. As indicated in **Figure 5D**, ZO-1 expression was significantly decreased after TBI ($P = 0.001$ compared with the sham + saline group and $P = 0.001$ compared with the sham + MB group), whereas MB treatment prevented the decrease in ZO-1 ($P = 0.004$).

MB Treatment Attenuates Neurological Deficits Caused by TBI

The restoration of spatial memory was evaluated by the number of crosses into the platform quadrant and the percentage of time spent in the platform quadrant in 60-s intervals. In the TBI + saline group, the time percentage of time spent in the platform quadrant was 37.29% ± 9.99%, which was significantly lower than that spent by the sham + saline group (64.59% ± 10.30%) ($P = 0.01$), the Sham + MB group (69.22% ± 15.86%)

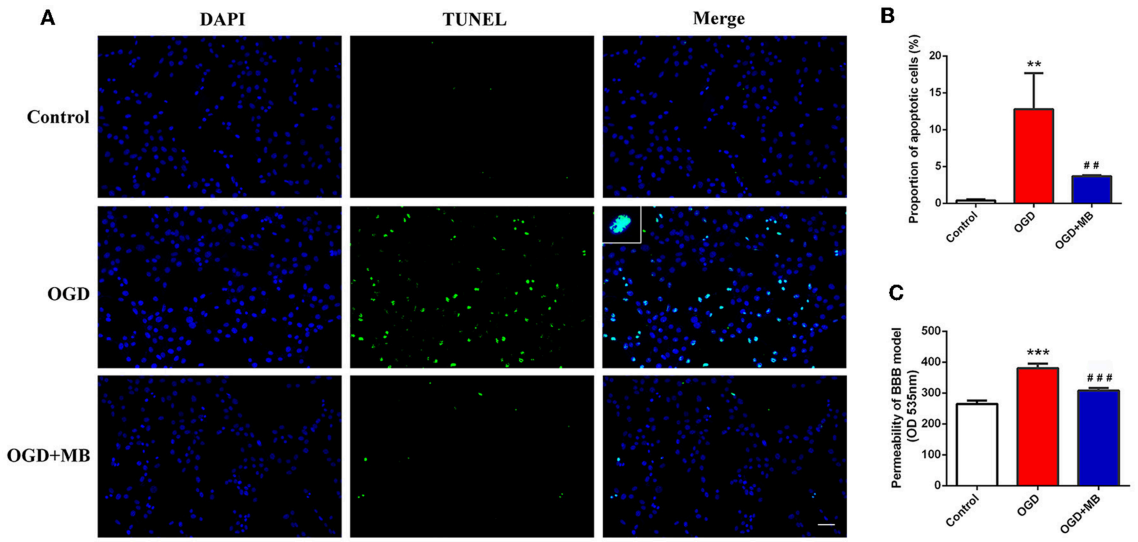

FIGURE 2 | Methylene blue (MB) treatment decreased the neuronal apoptosis and blood-brain barrier (BBB) permeability caused by oxygen glucose deprivation/reoxygenation (OGD) injury. **(A)** Representative fluorescence of apoptotic neurons. Fluorescence colors: terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL): green, DAPI: blue. Scale bar = 200 μm. TUNEL and DAPI double stained cells represented the apoptotic neurons. **(B)** Quantification of apoptotic neurons between the different groups. **< 0.01, vs. Control group, ## < 0.01, vs. OGD group. **(C)** Quantification of BBB permeability (OD 535 nm). ***< 0.001, vs. Control group, ### < 0.001, vs. OGD group.

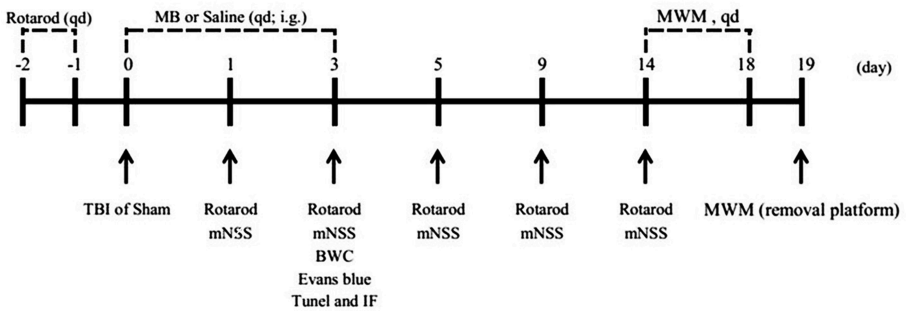

FIGURE 3 | Timeline of the animal experiments.

($P = 0.006$), and the TBI + saline group (64.06% ± 21.55%) ($P = 0.016$). The results of the number of crosses into the platform quadrant were consistent with those of the percentage of time spent in the platform quadrant. In the TBI + saline group, the number of crosses into the platform quadrant was 3.40 ± 1.82, which was significantly lower than that of sham + saline group (6.20 ± 1.92) ($P = 0.011$), the sham + MB group (6.75 ± 0.96) ($P = 0.005$), and the TBI + saline group (5.75 ± 0.90) ($P = 0.035$). However, there was no significant difference in either the percentage of time spent in the platform quadrant or the number of crosses into the platform quadrant between the sham + saline group, sham + MB group, and TBI + MB group (**Figures 6A–C**). These results demonstrate that MB treatment can improve TBI-induced cognitive deficits.

Motor dysfunction and recovery were assessed using the rotarod test. Before TBI induction, rotarod test performance was not significantly different between the TBI + saline group and TBI + MB group ($P = 0.832$). One day and 3 days after TBI, no significant difference in the time spent on the cylinder between the TBI + saline group and the TBI + MB group was observed ($P = 0.851$ and $P = 0.964$). Five days, 9 days, and 14 days after TBI, the time spent on the cylinder by the TBI + saline group was significantly lower than that spent by the TBI + MB group ($P = 0.004$, $P = 0.002$, and $P < 0.001$, respectively). These results suggest that MB treatment can improve the motor dysfunction caused by TBI (**Figure 6D**).

As depicted in **Figure 6E**, the mNSS were significantly increased after TBI. On the third day, the neurological function of the mice in the TBI + MB treatment group gradually recovered, and the mNSS were significantly lower than those of the TBI + saline group on days 3 ($P = 0.017$), 5 ($P = 0.01$), 9 ($P = 0.022$), and 14 ($P = 0.004$).

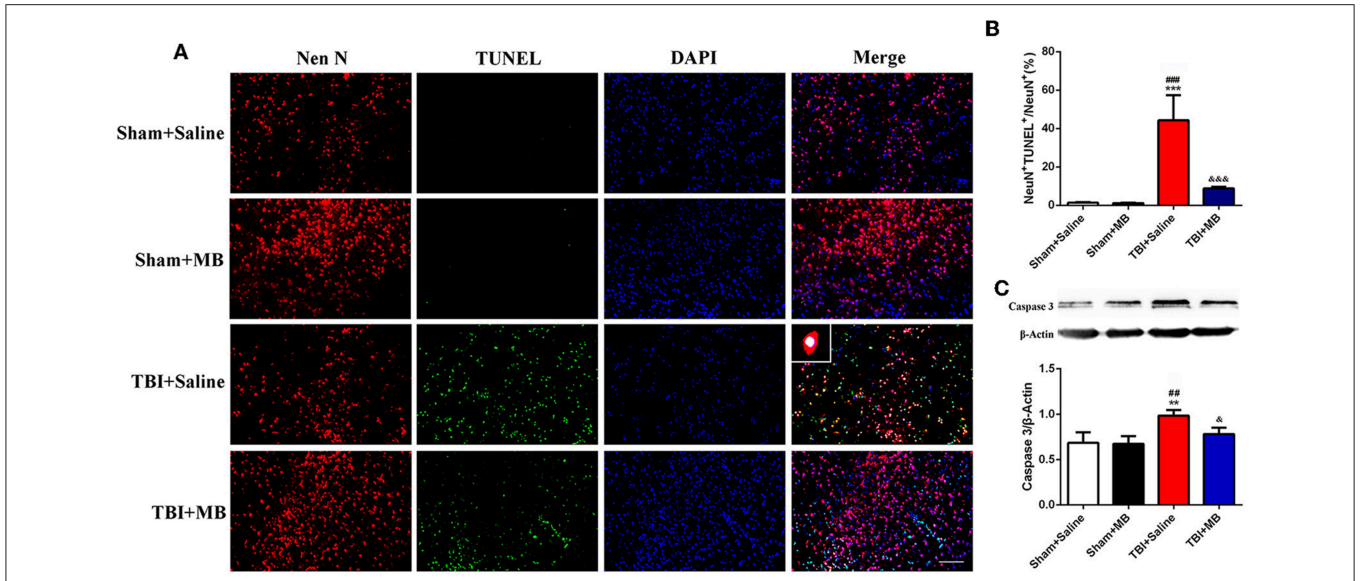

FIGURE 4 | (A) Representative fluorescence of apoptotic neurons in the cortex of peri-lesion at 3 days after TBI. Fluorescence colors: NeuN: red, terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL): green, and DAPI: blue. Scale bar = 400 μm. NeuN and TUNEL double stained cells represented the apoptotic neurons. **(B)** Quantification of apoptotic neurons between the different groups. ***< 0.001, vs. Sham + Saline group, ###< 0.001, vs. Sham + MB group, &&&< 0.001, vs. TBI + Saline group; **(C)** Representative images and quantitative analysis of caspase 3 expression. **< 0.01, vs. Sham + Saline group, ##< 0.01, vs. Sham + MB group, &< 0.05, vs. TBI + Saline group.

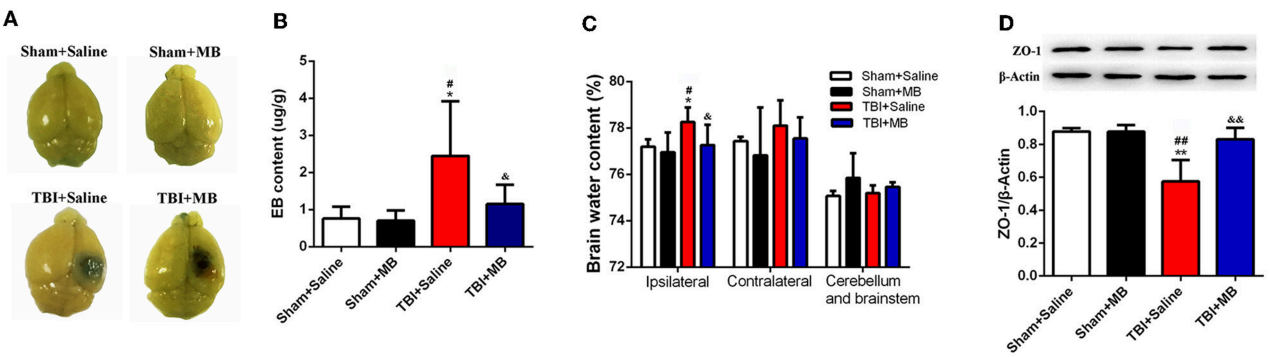

FIGURE 5 | (A) Representative images of brain tissues 1 h after Evans blue (EB) injection. **(B)** Quantification of EB content. *< 0.05, vs. Sham + Saline group, #< 0.05, vs. Sham + MB group, &< 0.05, vs. TBI + Saline group, $n = 46$/group. **(C)** Quantitative analysis of brain water content of ipsilateral, contralateral, cerebellum, and brainstem in different groups. *< 0.05, vs. Sham + Saline group, #< 0.05, vs. Sham + MB group, &< 0.05, vs. TBI + Saline group, n = 46/group. **(D)** Representative images and quantitative analysis of ZO-1 expression. **< 0.01, vs. Sham + Saline group, ##< 0.01, vs. Sham + MB group, &&< 0.01, vs. TBI + Saline group.

DISCUSSION

The cascades involved in secondary brain damage following TBI mainly occur in the mitochondria, the dysfunction of which mediates Ca^{2+} overload, cellular excitotoxicity, the release of ROS, and cell apoptosis (9). In addition, the processes of oxidative phosphorylation and ATP generation mainly occur in the mitochondria, which provide ∼95% of ATP (25). Once mitochondria become dysfunctional, the production of energy needed for the repair of damaged cells is reduced; this may aggravate cell damage. Therefore, mitochondrial dysfunction plays a pivotal role in the pathological processes of secondary brain damage following TBI, and mitochondria-targeted treatment of TBI may have the potential to improve the prognosis of TBI. Zhu et al. demonstrated that TBI animals treated with SS-31, a mitochondria-targeted peptide, exhibited obviously improved mitochondrial function and reduced secondary brain injury (25).

In the present study, we first detected the effect of MB on mitochondrial function *in vitro* using an OGD model in PC-12 cells. The results revealed that MB treatment can reduce neuronal ROS production, stabilize the neuronal MMP, and increase

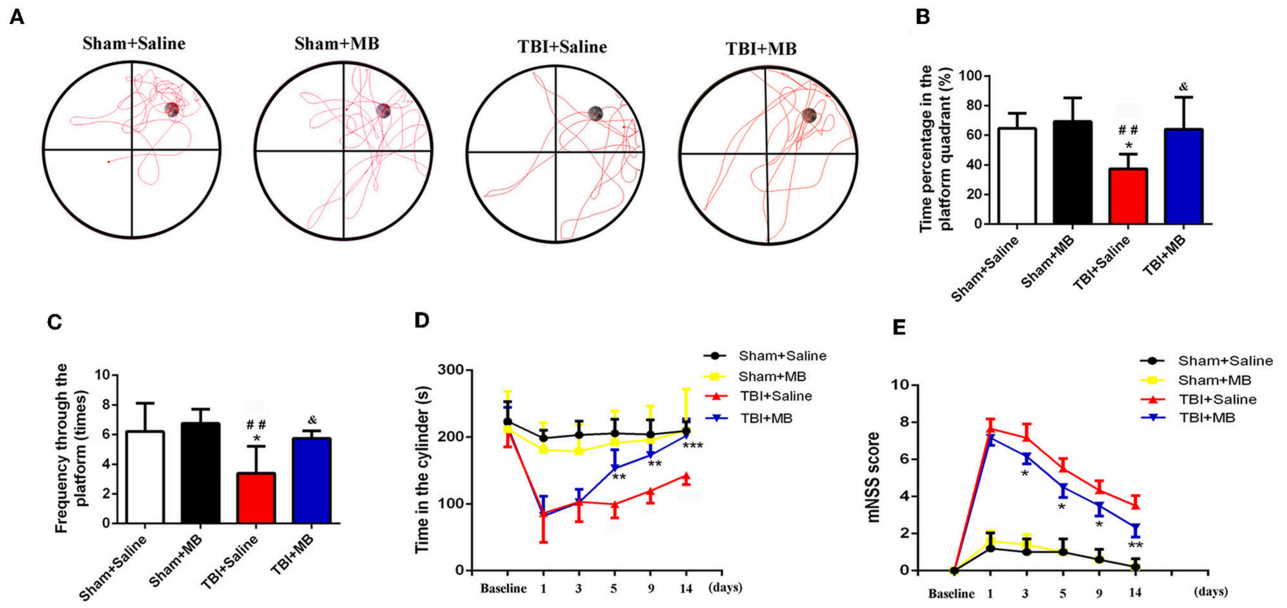

FIGURE 6 | (A) Representative images of Morris water maze in different groups, $n = 46$/group. **(B)** Time percentage of the mice in the platform quadrant between different groups. *< 0.05, vs. Sham + Saline group, ## < 0.01, vs. Sham + MB group, & < 0.05, vs. TBI + Saline group; **(C)** Frequency through the platform of the mice in the different groups. *< 0.05, vs. Sham + Saline group, ## < 0.01, vs. Sham + MB group, & < 0.05, vs. TBI + Saline group; **(D)** The duration time in the cylinder in the different groups at different time points. **< 0.01, vs. TBI + Saline group, ***< 0.001, vs. TBI + Saline group, $n = 46$/group; **(E)** mNSS Score of mice in the different groups at different time points. *< 0.05, vs. TBI + Saline group, **< 0.01, vs. TBI + Saline group, $n = 46$/group.

ATP production, suggesting that MB treatment can reverse the mitochondrial dysfunction caused by OGD injury. However, we did not determine mitochondrial function in neurons in tissue samples from the TBI + saline group and the TBI + MB group because it is difficult to distinguish mitochondria from neurons and from other cells in injured brain tissue.

Second, we demonstrated that the administration of MB in TBI mice can reduce neuronal apoptosis and improve BBB integrity both *in vitro* and *in vivo*. Mitochondria play a pivotal role in neuronal apoptosis after TBI. Under ischemia and hypoxia injury, cytochrome c is released from the mitochondrial membrane and binds with ATP and apoptotic protease activating factor (Apaf-1) to generate apoptosome complexes, which cleave procaspases to activate caspase 3 and induce neuronal apoptosis (26). In addition, the release of ROS and the reduction in ATP production also contribute to neuronal apoptosis (9, 27). MB can transfer electrons from NADH to cytochrome c and increase the stability of cytochrome c. This process increases ATP generation and reduces cytochrome c release and ROS production (13). Therefore, MB can reduce neuronal apoptosis after TBI.

The BBB is composed of pericytes, astrocytes, endothelial cells, and tight junction proteins and is surrounded by neurons (28, 29). After TBI, the release of ROS directly downregulates the expression of tight junction proteins, such as ZO-1 (4). On the other hand, astrocytes transfer their mitochondria to damaged neurons to rescue them (30). These processes directly lead to an increase in the permeability of the BBB. However, neuronal apoptosis also participates in BBB damage. MB treatment can reverse these processes and thus maintain BBB integrity. Moreover, Fenn and his colleagues (20) demonstrated that MB treatment has an anti-inflammatory effect in TBI and that MB treatment directly increases the expression of IL-10 and reduces IL-1β expression in microglia, which can also attenuate inflammatory-mediated BBB damage.

In TBI animals, treatment with MB not only improved cognitive and motor function caused by TBI but also significantly improved overall neurological function. This result was very similar to that of previous studies. Talley Watts et al. (19, 21) showed that MB can minimize neuronal degeneration, behavioral deficits, and lesion volume in TBI animals. Zhao et al. (22) demonstrated that MB exerts a neuroprotective effect in TBI by inhibiting microglial activation, decreasing brain edema, and increasing autophagy. However, while these studies focused on the anti-inflammatory effects of MB in TBI, they did not focus on BBB integrity and neuronal apoptosis, making them quite different from our research. Based on previous studies, MB has three main functions in TBI, namely, exerting anti-inflammatory effects, improving BBB integrity, and reducing neuronal apoptosis, indicating that MB is a potential drug for improving the prognosis of TBI.

Mitochondrial dysfunction following TBI is also involved in many other secondary damages. Synaptic mitochondria are essential for maintaining synaptic plasticity and normal neurotransmission, which dysfunction may lead to neurodegeneration. While synaptic mitochondria have been shown to suffer more injury than non-synaptic mitochondria in a TBI model (31). This reveals that MB improves the spatial memory of TBI mice perhaps partly by improving synaptic

mitochondrial function. Additionally, mitochondria can also crosstalk with miRNAs involved in cellular cascade responses to TBI (32), indicating that MB may regulate microRNAs in TBI.

Repurposing old drugs is recommended by modern pharmacology. For example, MB has been used in clinical practice for nearly 130 years, and its safety and side effects have been well-established. Compared with developing new drugs for TBI therapy, repurposing MB has the advantages of saving money and time, and it can be rapidly applied clinically (33). However, although MB can improve the prognosis of TBI mice, the existing research results are not enough to support the immediate use of MB in TBI patients. The other therapeutic effects of MB in secondary brain injury still require investigation in the future.

CONCLUSIONS

This study provides evidence that MB can reverse neuronal mitochondrial dysfunction caused by OGD injury. Both *in vitro* and *in vivo*, MB treatment can reduce neuronal apoptosis and improve BBB integrity. In TBI animals, treatment with MB not only improves cognitive and motor function caused by TBI but also significantly improves overall neurological function. Our findings suggest that MB is a potential candidate for the treatment of TBI. Future research should focus on other therapeutic effects and mechanisms of MB in secondary brain injury.

DATA AVAILABILITY STATEMENT

All data that support the results of this study are available from the first author upon request.

ETHICS STATEMENT

All animal experimental procedures in this study were approved by the Tianjin Medical University Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

JZ and LY designed the study. JS, QL, YG, and WX carried out the experiments. JS, QL, and YG interpreted the results, carried out statistics analysis, and prepared the figures. JS prepared the manuscript. JZ supervised the study and revised the manuscript. All the coauthors listed approved the manuscript.

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ORIGINAL RESEARCH
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A Clinical Study of the Intra-Neuroendoscopic Technique for the Treatment of Subacute-Chronic and Chronic Septal Subdural Hematoma

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Objective: The surgical technique, safety, efficacy, and clinical application value of the intra-neuroendoscopic technique (INET) for the treatment of subacute-chronic and chronic septal subdural hematoma was investigated based on the structure and pathological features of the hematoma wall, and the critical factors of hematoma growth and recurrence were determined, in order to provide reference for clinical drug treatment.

Methods: This was non-randomized concurrent control study. A total of 94 patients who met the inclusion criteria were recruited between May 2015 and February 2019 and were divided into the INET treatment group (INET group, 45 cases) and the burr hole drainage (BHD) treatment group (control group, 49 cases). The hematoma fluid components and the morphological structure and pathological characteristics of the hematoma wall were analyzed, and the surgical duration, subdural drainage tube (SDT) placement duration, intracranial infection rate, Bender grade at the 1 month post-operative follow-up and hematoma recurrence rate within the 6 months of post-operative follow-up were compared between the two groups. A multiple logistic regression model was established to analyze the risk factors associated with recurrence within 6 months.

Results: Intraoperative endoscopy showed that the adhesion bands that formed early in the hematoma cavity were strip-like and that those that formed late were lock-column-like. The hematoma cavity was divided into different-sized chambers with by these strips/columns. Pathological sections of cyst wall reveled angiogenesis inside the cyst and mucus-like changes, rupture and hemorrhage in the vascular wall. Obvious inflammatory cell infiltration and fibrous connective tissue hyperplasia were observed in the cyst wall. The osmotic pressure of the hematoma fluid was not significantly different from that of the peripheral venous blood [(296.7 ± 10.3) mOsm/kg vs.

(291.5 ± 12.4) mOsm/kg, $p = 0.68$]. However, the D-dimer contents which reflect the severity of fibrinolysis in the hematoma and the proinflammatory cytokine interleukin 6 (IL-6) were significantly higher in the hematoma fluid than in the peripheral venous blood. The surgery duration for the INET group was significantly longer than that for the control group [(60.4 ± 10.6) min vs. (44.1 ± 9.8) min, $p = 0.00$], but both the hematoma recurrence rate within 6 months of post-operative follow-up (4.4 vs. 24.5%, $p = 0.00$) and the SDT placement duration [(2.1 ± 0.6) d vs. (3.9 ± 0.7) d, $p = 0.00$] for the INET group were both lower than those for the control group. The intracranial infection rate did not differ significantly between the two groups (4.4 vs. 10.2%, $p = 0.50$). The overall effective rate of the Bender grade at 1 month of follow-up did not differ significantly between the two groups (95.6 vs. 87.8%, $p = 0.32$), but the proportion of patients who recovered to Bender grade 0 with no symptoms was significantly higher in the INET group than in the control group (86.7 vs. 67.3%, $p = 0.03$). Multiple logistic regression analysis showed that INET surgery [odds ratio (OR) 3.71, 95% confidence interval (CI) 1.31–9.62, $p = 0.02$], age of 65 years or younger (OR 1.51, 95% CI 1.05–2.87, $p = 0.03$) and unilateral subdural hematoma (OR 1.76, 95% CI 1.05–3.41, $p = 0.02$) were independent factors that reduced the post-operative recurrence rate.

Conclusion: The INET surgical plan based on the structure and pathological features of the subacute-chronic and chronic subdural hematoma wall can reduce the recurrence rate and improve the clinical prognosis.

Trial registration: ClinicalTrials.gov, NCT02515903. Registered 5 August, 2015.

Keywords: intra-neuroendoscopic technique (INET), transparent sheath, chronic subdural hematoma (CSDH), inflammatory factor, pathology, prognosis

— a surgical treatment plan based on the structure and pathological features of the subacute-chronic and chronic subdural hematoma wall that reduces the recurrence rate and improves patient prognosis

INTRODUCTION

Chronic subdural hematoma (cSDH) often affects middle-aged and elderly individuals with a history of minor trauma and is a common intracranial hemorrhagic disease, accounting for approximately 10% of all intracranial hematomas. Currently, the most preferred treatment for cSDH is still unclear (1). The incidence of cSDH has been increasing. According to the latest reports (2–4), the incidence of cSHD among individuals older than 65 years has increased from 8–18/100,000 15 years ago to 48/100,000. The recurrence rate of hematoma after different surgeries in patients with cSDH is 2.3–38.7% (3, 5–8). Burr hole drainage (BHD) is still a classic surgical procedure for treating cSDH and is a simple and effective method for alleviating early brain compression symptoms. However, the rate of patients requiring reoperation due to post-operative recurrence is as high as 31.6% (3). Subacute-chronic and chronic septal subdural hematomas are complicated cSDH types with high post-operative recurrence rates. Because a large number of recurrent patients are elderly and often accompanied with multiple organ or systemic disease, they are unable to tolerate highly traumatic craniotomy, which makes these recurrent cases difficult to treat clinically. Based on the structure and pathological features of the subacute-chronic and chronic septal subdural hematoma wall, the intra-neuroendoscopic technique (INET) was performed in this non-randomized concurrent control study (classic BHD was the control treatment), and the surgical technique, safety, efficacy, and clinical value of INET for the treatment of these diseases were investigated. The key factors of hematoma growth and recurrence were analyzed through clinical and pathological examinations, aiming to provide a reference for the clinical treatment of cSDH.

MATERIALS AND METHODS

General Data

A total of 94 patients who met the inclusion criteria were recruited between May 2015 and February 2019 from Shenzhen People's Hospital and Nanfang Hospital. The patients were divided into two groups according to the preferences of the patients and their families: 45 patients in the INET group and 49 patients in the control group. The two groups did not differ significantly in sex, average age, midline shift, pre-operative hematoma size, pre-operative Bender grade, and medical history (such as definitive trauma, anticoagulant drugs, hypertension, diabetes, and stroke) ($P > 0.05$); therefore, the two groups were comparable. However, the two groups differed significantly in the

proportions of patients aged 65 years and younger and patients who had a unilateral subdural hematoma ($P < 0.05$) (**Table 1**).

All patients underwent cranial computed tomography (CT) to confirm the diagnosis. The hematoma volume was measured according to the Coniglobus formula (9), and the severity of the midline shift was determined by two senior physicians who averaged the measurements of the layer with the maximum midline shift on CT images. This study was approved by the ethics committee of the hospital (approval No. NFEC-2015-034) and was registered as clinical research at ClinicalTrials.gov (NCT 02515903).

Case Inclusion and Exclusion Criteria

Inclusion Criteria

(1) Adults aged <85 years, (2) patients with clear clinical symptoms but were not comatose and did not have dilated pupils, and their Bender grade was I-III, (3) CT upon admission showed that the patients had subacute-chronic subdural hematoma, or the imaging revealed chronic septal subdural hematoma, compressed brain tissue and shifted midline structure, and (4) the patients or their family agreed to enter the clinical study and allowed follow-up observations.

Exclusion Criteria

(1) CT upon admission showed that the patients had acute subdural hematoma, (2) patients with severe systemic diseases, such as severe dysfunctions of the heart, liver, lung and kidney, and (3) patients with abnormal blood coagulation, such as long-term use of anticoagulant drugs or coagulopathy caused by abnormal blood coagulation.

Research Methods and Observation Indicators

This was a non-randomized concurrent control study. There were three experienced doctors in our center to perform this operation, they have all undergone rigorous training. The investigators thoroughly explained the advantages and disadvantages, surgical risks, remedial measures, treatment costs and potential prognostic outcomes of the two techniques to each subject who met the inclusion and exclusion criteria. The patients and their family chose to join either group according to their preferences. After enrollment, the scalp incision length, bone hole diameter, surgery duration, subdural drainage tube (SDT) placement duration, intracranial infection rate during hospitalization, Bender grade of the patients at the 1 month post-operative follow-up and hematoma recurrence rate at the 6 month post-operative follow-up were compared between the two groups. For patients in the INET group, the hematoma cyst wall was taken during the operation for pathological examination, and the hematoma fluid and peripheral venous blood were taken to compare the osmotic pressure and IL-6 and D-dimer levels. During the study, patients were given 20 mg of atorvastatin orally, once a day for 3 months. The Bender grade criteria were as follows: Grade 0—no symptoms; Grade I—general symptoms, such as dizziness and headache, no unconsciousness, no psychiatric symptoms, and no obvious focal neurological deficits; Grade II—lethargy or confusion, psychiatric symptoms, and mild focal neurological deficits; Grade III—stupor, obvious psychiatric symptoms and focal neurological deficits; and Grade IV—coma or signs of herniation.

TABLE 1 | Comparison of pre-operative baseline data between the INET and control groups.

Baseline indicator	INET group ($n = 45$)	Control group ($n = 49$)	*p*-value
Sex (Male/Female)	29/16	30/19	0.75
Age (Years)	73.2 ± 5.5	70.6 ± 6.1	0.17
Age≤65 years [n(%)]	9 (20.0%)	20 (40.8%)	0.03
A definitive history of trauma [n(%)]	37 (82.2%)	34 (69.4%)	0.15
Unilateral subdural hemorrhage [n(%)]	38 (84.4%)	32 (65.3%)	0.03
Midline shift (mm)	9.6 ± 3.1	8.8 ± 3.8	0.52
Hematoma volume (ml)	96.8 ± 19.2	104.3 ± 21.3	0.36
Use of anticoagulant/antiplatelet drugs [*n* (%)]	23 (51.1%)	30 (61.2%)	0.32
Hypertension	32 (71.1%)	28 (57.1%)	0.16
Diabetes	22 (48.9%)	30 (61.2%)	0.23
Stroke	17 (37.8%)	14 (28.6%)	0.47
Bender grade			
Grade I	9 (20.0%)	15 (30.6%)	0.24
Grade II	24 (53.3%)	26 (53.1%)	0.67
Grade III	12 (26.7%)	8 (16.3%)	0.22

INET

Instruments

The INET equipment in this study was composed of a high-definition imaging system, cold light source, Zeppelin large working channel endoscope, transparent sheath, hematoma smashing aspirator, endoscopic scissors, endoscopic tweezers, endoscopy biopsy forceps, and endoscope specialized bipolar electrocoagulator. The large working channel endoscope (NEH 0/30-177-6.5) had a working length of 177 mm, external diameter of 6.5 mm, and view angle of 0 or 30°. The working channel diameter was 3.7 mm, and the diameters of the two suction/flushing channels were both 1.5 mm (10–12). The application of INET relies on a novel product developed by our team-the transparent sheath (Chinese Patent No. ZL 200820046232.0, State Intellectual Property Office of P.R. China, website: http://cpquery.sipo.gov.cn), which can fit seamlessly on a Zeppelin large channel endoscope. The transparent sheath has an external diameter of 7.7 mm, is colorless and transparent and has a transparent tip that can be removed together with the endoscope as well as a scale and a fixation device (**Figure 1**).

Surgical Method

During the surgery, all patients were under general anesthesia with tracheal intubation. The surgical incision was placed in the middle of the largest hematoma level, normally at the corner of the hematoma located 3–4 cm in front of the apical nodule. The incision, with a length of 4–5 cm, was parallel to the scalp vessels. The incision was either straight or "S" shaped (**Figures 2a,b**). After a hole was drilled in the skull, a bone flap (diameter, 2.0–3.0 cm) was cut using a milling cutter (**Figure 2c**). The wall layer of the hematoma sac was observed by a cross-shaped incision after lifting the dura mater (**Figure 2d**). The hematoma

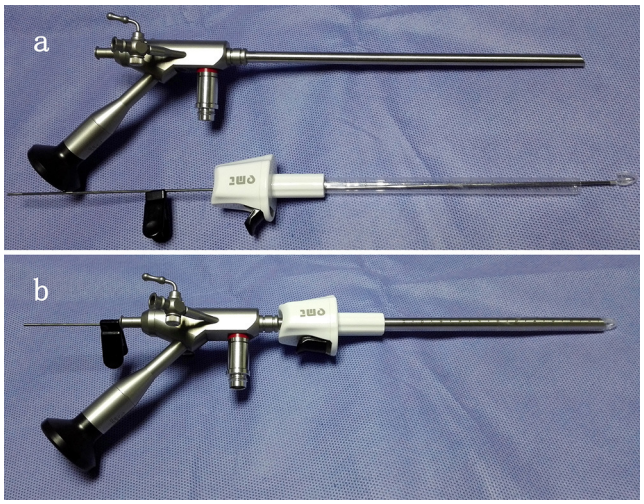

FIGURE 1 | Transparent sheath and neuroendoscope before **(a)** and after **(b)** assembly.

fluid was first extracted by puncture, and peripheral venous blood was taken simultaneously; both samples were sent for biochemical analyses, and the sac wall was sent for pathological examination. The endoscope was placed and was continuously flushed until clear. The hematoma fluid and old blood clots were removed through the endoscope working channel under alternating water and air environments, and the hemorrhage site was electrocoagulated to stop bleeding (**Figure 3**). The hematoma septal strips/columns were resected under the endoscope, and the blood clots in the septal chambers were removed (**Figure 4**). After cleaning the hematoma cavity, part of the hematoma wall and visceral capsule were removed from the bone window; the extent of the capsule resection was larger than 5.0 × 5.0 cm so that the hematoma cavity was fully connected to the subdural cavity (**Video 1**). After the surgery, the operation area was cleaned, and a drainage tube was visually placed in the subdural hematoma cavity. The small bone flap was fixed, the scalp was sutured layer by layer, and the skull was closed. Cranial CT was performed 24 h after surgery.

Statistical Analysis and Sample Size Estimation

Statistical Analysis

The data were managed and analyzed using Stata 13.1 software. A multiple logistic regression model was established, and the factors that showed intergroup differences and surgical methods were included in the regression model to screen for independent factors that affected the outcome. The *t*-test was performed to determine differences in age, surgical duration, SDT placement duration, midline shift, and hematoma volume between two groups. The χ^2 test and Fisher's exact probability test were used to compare the rates between two groups. The results for the quantitative data are expressed as the mean ± standard deviation. Endpoint event was defined as whether the subdural hematoma recurred by the 6 month follow-up. Patients who left the study, were lost to follow-up or died were exclude from the study. $P < 0.05$ was considered statistically significant.

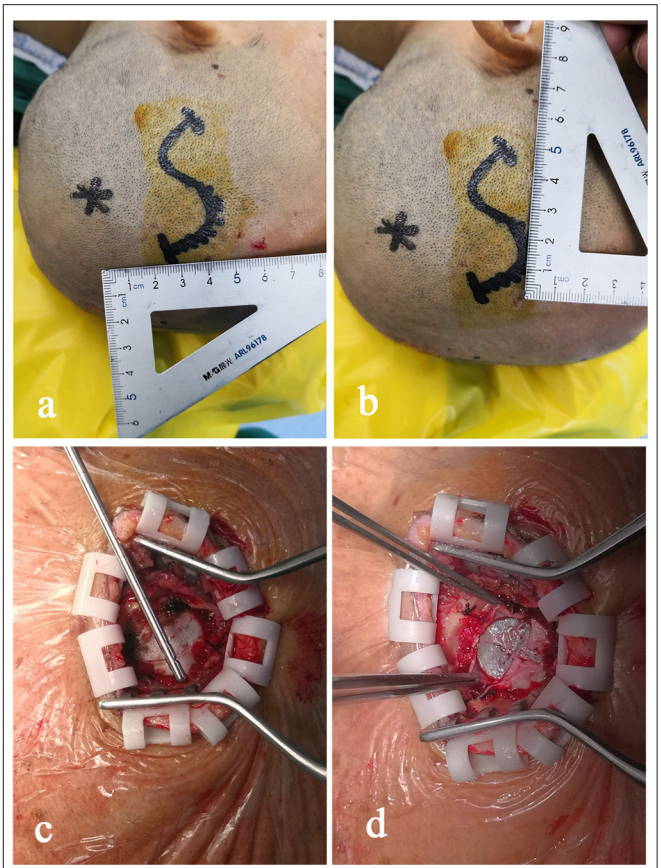

FIGURE 2 | The midpoint of the surgical incision is generally 3–4 cm in front of the apical nodule. The direction of the incision is parallel to the scalp vessel, and the incision is in an "S" shape with a length of 4–5 cm **(a,b)**. After a hole is drilled in the skull, a small bone flap with a diameter of 2.0–3.0 cm is cut using a milling cutter **(c)**. The wall envelop of the hematoma sac can be seen using a cross-shaped incision after lifting the dura mater **(d)**.

Sample Size Estimation

The sample size was estimated based on the recurrence rate of the pilot experiment with α = 0.05 (according to the reference table, u0.05/1 = 1.96, bilateral), ß = 0.10 (according to the reference table, u0.10 = 1.282, bilateral) and power=1-ß = 0.9. In the pilot experiment, the recurrence rate within 6 months after surgery for the INET group was Pe = 2.7%, the rate for the control group was Pc = 26.9%, and P = (Pe+Pc)/2=0.148, k = 1. The calculation indicated that at least 45 patients were required for each group.

Sample size estimation formula:

$$N = \frac{(u_\alpha + u_\beta)^2(1 + 1/k)p(1-p)}{(p_e - p_c)^2}$$

RESULTS

Baseline Indicators and Clinical Indicators

The pre-operative baseline indicators, such as sex, average age, severity of midline shift, pre-operative hematoma volume, pre-operative Bender grade, and medical history (such as definitive

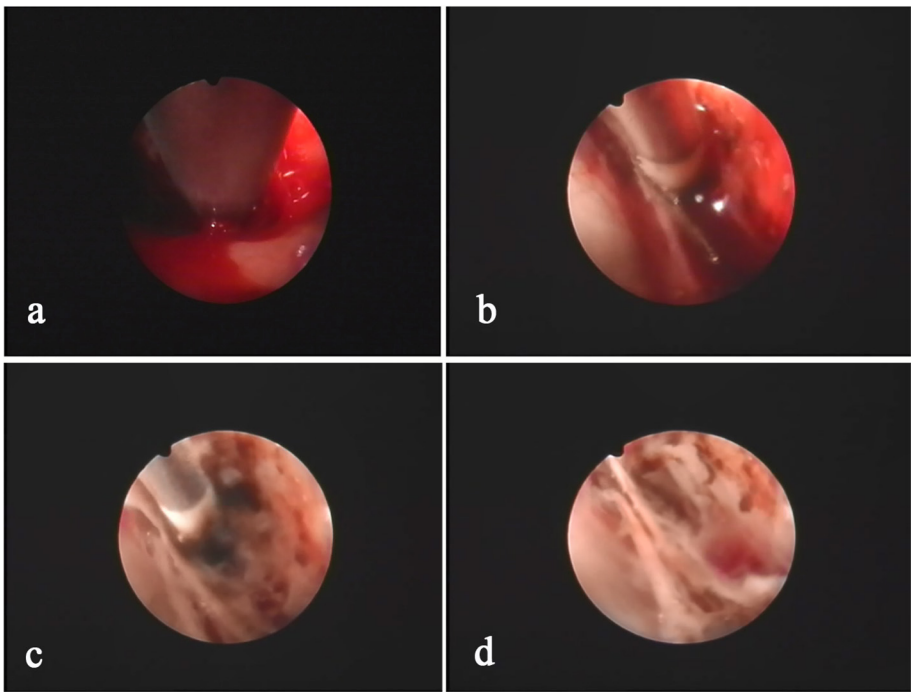

FIGURE 3 | The hemorrhage sites inside the hematoma cavity are usually located at the folding point of the visceral layer and wall layer of the sac. The hemorrhage site **(a)** is identified after clearing the hematoma using an aspirator. Bleeding is precisely stopped using a specialized bipolar electrocoagulator on the endoscope **(b,c)**. After hemostasis, the hematoma cavity is washed with warm saline to confirm hemostasis **(d)**.

trauma, anticoagulant drugs, hypertension, diabetes and stroke), did not differ significantly between the two groups ($p > 0.05$). However, the proportion of patients at an age of 65 years or younger (20.0 vs. 43.6%, $p = 0.03$) and the proportion of patients with unilateral subdural hematoma (91.4 vs. 69.2%, $p = 0.02$) differed significantly between the two groups (**Table 1**). The surgical incision length [(4.0–5.0) cm vs. (3.0–3.5) cm] and surgery duration for the INET group were significantly longer than those for the control group [(60.4 ± 10.6) min vs. (44.1 ± 9.8) min, $p = 0.00$], but the SDT placement duration [(2.1 ± 0.6) d vs. (3.9 ± 0.7) d, $p = 0.00$] and the hematoma recurrence rate at the 6 month follow-up (4.4 vs. 24.5%, $p = 0.00$) for the INET group were significantly lower than those for the control group. The intracranial infection rate (4.4 vs. 10.2%, $p = 0.50$) and the overall Bender grade effective rate at the 1 month follow-up (95.6 vs. 87.8%, $p = 0.32$) did not differ significantly between the two groups, but the proportion of patients who recovered to Bender grade 0 with no symptoms was significantly higher in the INET group than that in the control group (86.7 vs. 67.3%, $p = 0.03$) (**Table 2**).

Biochemical and Pathological Examination Indicators

Simultaneous examination of the hematoma fluid and peripheral venous blood showed that the osmotic pressure did not differ significantly between the two groups [(296.7 ± 10.3) mOsm/kg vs. (291.5 ± 12.4) mOsm/kg, $p = 0.68$]. The level of the proinflammatory cytokine interleukin 6 (IL-6) [(58.6 ± 14.6) pg/ml vs. (3365.8 ± 863.7) pg/ml, $p = 0.00$] and the content of D-dimer [(2044.5 ± 218.3) ng/ml vs. (1244236.8 ± 152545.6) ng/ml, $p = 0.00$], which reflects the local severity of fibrinolysis in the hematoma, were significantly higher than those in the peripheral venous blood (**Table 3**). Pathological examination of the hematoma wall capsule indicated angiogenesis inside the capsule that was accompanied with mucus-like changes and rupture of the newly generated vessels resulting in hemorrhage. Neutrophil and lymphocytic infiltration and fibrous connective tissue hyperplasia were observed in the hematoma capsule (**Figure 5**).

Multiple Logistic Regression Analysis

Logistic regression analysis showed that INET surgery [odds ratio (OR) 3.71, 95% confidence interval (CI) 1.31–9.62, $p = 0.02$], age of 65 years or younger (OR 1.51, 95% CI 1.05–2.87, $p = 0.03$) and unilateral subdural hematoma (OR 1.76, 95% CI 1.05–3.41, $p = 0.02$) were independent factors that reduced the post-operative recurrence rate (**Table 4**).

DISCUSSION

Brain atrophy in middle-aged and elderly individuals in a natural state leads to expansion of the subarachnoid space and increased brain tissue activity, and the bridging veins become full and relatively prolonged; therefore, mild craniocerebral trauma is sufficient to cause drainage of the convex surface of the brain to the subdural bridging veins or rupture of the small veins

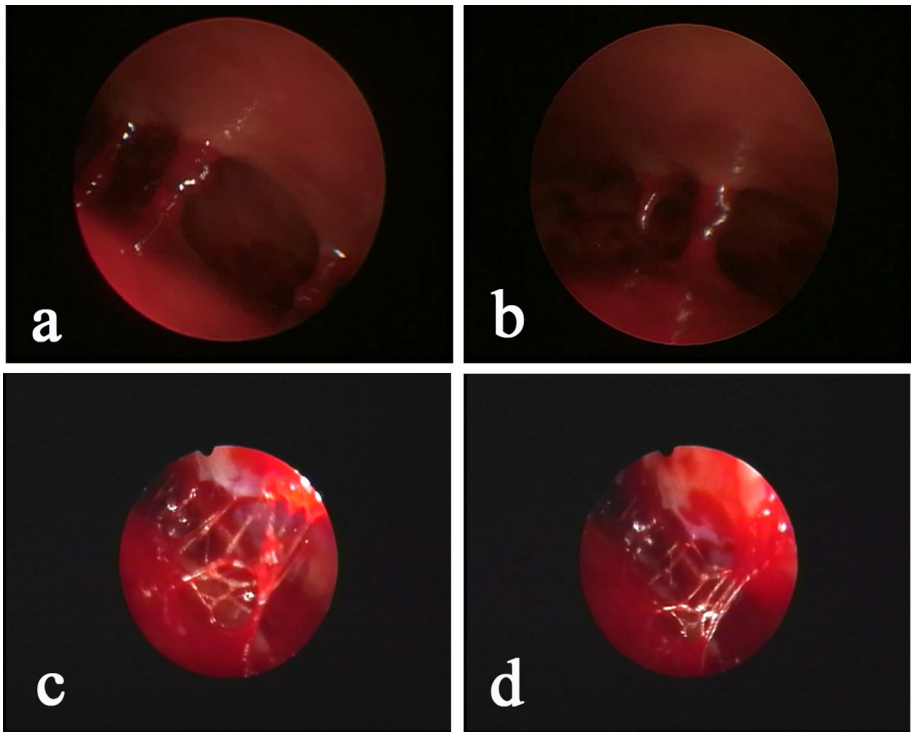

FIGURE 4 | Neuroendoscopic observation of the hematoma with "separation," as determined by pre-operative CT, shows that the "separation" is mainly a separation lock column **(a,b)** or separation strip **(c,d)** instead of a real closed septum. The relative separation is sufficient to block the blood clot in the chamber. Conventional BHD is not sufficient to completely drain the hematoma, but INET is able to remove the separation strip or separation lock column and visually clean the old blood clots.

TABLE 2 | Comparison of intraoperative and post-operative clinical indicators between the INET and control groups.

Clinical indicator	INET group ($n = 45$)	Control group ($n = 49$)	*p*-value
Surgery duration (min)	60.4 ± 10.6	44.1 ± 9.8	0.00
SDT placement duration (d)	2.1 ± 0.6	3.9 ± 0.7	0.00
Intracranial infection rate	2 (4.4%)	5 (10.2%)	0.50
Recurrence rate (6 months)	2 (4.4%)	12 (24.5%)	0.00
Overall effective rate	95.6%	87.8%	0.32
Bender grade (1 month)			
Grade 0	39 (86.7%)	33 (67.3%)	0.03
Grade I	5 (11.1%)	11 (22.4%)	0.14
Grade II	1 (2.2%)	5 (10.2%)	0.25

Continuous variables are presented as the mean ± standard deviation, and categorical variables are presented as count (percentage).

TABLE 3 | Comparison of test indicators between subdural hematoma fluid and peripheral venous blood.

Test indicator	Peripheral venous blood ($n = 94$)	Hematoma fluid ($n = 94$)	*p*-value
Osmotic pressure (mOsm/kg)	291.5 ± 12.4	296.7 ± 10.3	0.68
IL-6 (pg/ml)	58.6 ± 14.6	3365.8 ± 863.7	0.00
D-dimer (ng/ml)	2044.5 ± 218.3	1244236.8 ± 152545.6	0.00

adjacent to the sagittal sinus, leading to subdural hematoma (13). Surgery is the main treatment for cSDH, and common surgical methods include BHD and craniotomy. Currently, BHD is the main method and uses single or double holes. Some clinicians also inject urokinase into the hematoma cavity to accelerate blood clot efflux (2, 14). However, the recurrence rate after BHD is high in patients with subacute-chronic and chronic septal subdural hematomas. Our study showed that the hematoma recurrence rate within 6 months after surgery in the control group was as high as 24.5%, which is similar to the data reported in previous studies (15–17). Recent studies (18–21) have found that atorvastatin can reduce the recurrence rate of cSDH. To prevent intervention from this factor, all patients in this study were treated with 20 mg of atorvastatin once daily for 3 months. One study (22) showed that stopping hemorrhage at a new bleeding point of a subacute-chronic subdural hematoma is an important factor affecting post-operative recurrence, which can be better achieved in endoscopic surgery. In this study, we also found during surgery that almost all cases had obvious fresh bleeding at the transition of the visceral layer and wall layer of the hematoma sac that required electrocoagulation for hemostasis (**Figure 3**). Additionally, incomplete hematoma clearance during surgery is also an important cause of recurrence of post-operative hematoma (23, 24). Fibrin degradation products in residual blood clots can form hematoma capsules, and in the

wall layer capsules, small bold vessels continue to bleed so that the hematoma cavity expands constantly. In our study, pathological examination of the hematoma capsule in the INET group showed that there were many newly generated blood vessels in the capsule and that the vascular wall was accompanied by mucus-like changes, incomplete and partial rupture, and bleeding. The sac wall had mass inflammatory cell infiltration and fibrous connective tissue hyperplasia (**Figure 5**). During the INET surgery, we observed thin and soft old blood clots inside many of the separated cavities of the chronic septal subdural hematoma that needed to be completely cleared by opening the septal cavity under endoscope (**Figure 4**). Conventional BHD is unable to treat septal strips and columns; therefore, blood clots are not drained completely in a short period of time, which is one of the important factors causing subsequent recurrence. Examination of the hematoma fluid and peripheral venous blood showed that the osmotic pressure did not differ significantly between the two [(296.7 ± 10.3) mOsm/kg vs. (291.5 ± 12.4) mOsm/kg, $p = 0.68$], suggesting that osmotic pressure was not the main cause of increased hematoma among the patients in this study. However, we found that the proinflammatory cytokine IL-6 in the hematoma fluid was significantly higher than that in the peripheral venous blood [(3365.8 ± 863.7) pg/ml vs. (58.6 ± 14.6) pg/ml, $p = 0.00$]. IL-6 can increase the gap between vascular endothelial cells and thus increase the permeability of blood vessels, suggesting that the inflammatory response is an important factor that leads to hematoma growth and recurrence (25–27). Additionally, our study also showed that the D-dimer level in the subdural hematoma fluid was significantly higher than that in the peripheral venous blood [(1244236.8 ± 152545.6) ng/ml vs. (2044.5 ± 218.3) ng/ml, $p = 0.00$], suggesting that local fibrinolysis in the hematoma cavity was hyperactive and continuous hyperactivation of fibrinolysis could cause recurrent bleeding of the hematoma sac wall, which is also an important factor that leads to subdural hematoma growth and recurrence (28, 29).

The INET used in this study can completely clear old blood inside the hematoma cavity and precisely stop bleeding at the bleeding sites. INET can also remove the separation strips/columns formed inside the hematoma cavity after electrocautery so that the hematoma cavity is fully connected, which helps adequate drainage. We did not find an intact septum in the hematoma of patients with septal subdural hematoma, as indicated in the preoperative CT imaging. The separations shown in the imaging were mainly separation strips/columns observed under endoscopy (**Figure 4**), but these relatively incomplete separations were able to prevent sufficient drainage of the blood clots inside the septal cavities through BHD. During the treatment of cSDH using neuroendoscopy, Shiomi et al. (30) found that a trabecular structure was present in 65% of the hematoma cavities and that 30% of the patients had blood clots inside the hematoma cavities. Therefore, removing the separation strips and columns under endoscopy can remove the support from the hematoma cavities and can thoroughly clear blood clots. Sufficient drainage is an important means to prevent recurrence. There are numerous blood clots inside subacute-chronic mixed subdural hematomas, and after drainage and decompression, hematomas are prone to bleeding again. As a result, the effect of simple BHD is poor. In contrast, INET can fully clear blood clots and stop bleeding at fresh bleeding sites by electrocoagulation (**Figure 3**). During surgery, the flexible compatibility of INET in both water and air environments is an advantage. Hemostasis of large bleeding sites can be achieved under an air environment, and small bleeding sites can be effectively stopped in a water environment by continuous flushing with 39°C saline. Our study found that the hematoma recurrence rate at the 6 month follow-up was 4.4% in the INET group, which was significantly lower than that in the control group. The latest report of recurrence rate using soft neuroendoscopic techniques to treat chronic subdural hematoma was 5.33% during 0.5–8 years of follow-up, which was similar to ours (31). All the recurrent cases were patients with bilateral subacute-chronic mixed subdural hematomas. Our study found that the structural characteristics of the cSDH cavity are as follows: (1) The bleeding site inside the hematoma cavity is often at the wall layer, especially at the fold between the wall layer and the visceral layer close to the dura mater, and the main root of the fiber strip or column is also at the wall layer. (2) Fiber strings are first formed inside the cavity, followed by septal columns and the strips/columns divide the hematoma into multiple chambers with different sizes. (3) The visceral layer of the hematoma wall rarely contains any blood vessels, and it is significantly thinner than the wall layer. The hematoma visceral layer in a few cases is absent under neuroendoscopy, and the sac wall visceral layer capsule is undetectable. (4) Normally, the space between the visceral capsule of the hematoma and the arachnoid membrane is easily separated, and there is only a little adhesion. After opening the visceral capsule, the neuroendoscope can enter the space for exploration in the water environment (**Video 2**). The key points of the INET surgery designed based on the above characteristics include the following. (1) The blood in the hematoma cavity is completely removed visually and the separation strips and columns inside the hematoma cavity can be removed with the help of simple instruments. Precise electrocoagulation can be performed at the bleeding sites inside the hematoma cavity to stop bleeding, especially at the fold between the visceral and wall layers. (2) Part of the hematoma cavity wall layer and visceral lay capsule are resected during the surgery. In this study, the resection range of the capsule in all patients was more than 5 cm in diameter to ensure sufficient connection between the hematoma cavity and the subdural space. The hematoma cavity was completely removed and washed before resection, and harmful substances, such as blood clots, eosinophils, fibrinogen, and inflammatory factors, were cleared to prevent a sterile inflammatory reaction in the subdural space. The follow-up data indicated that the overall effective rates for Bender grade at the 1 month follow-up did not differ significantly between the two groups (95.6 vs. 87.8%, $p = 0.32$), but the proportion of patients who recovered to Bender grade 0 with no symptoms was significantly higher in the INET group than in the control group (86.7 vs. 67.3%, $p = 0.03$), suggesting that INET treatment is more effective in alleviating clinical symptoms and improving neurological functions. Although the intracranial infection rate did not differ significantly between the two groups, the SDT placement time of the five patients with intracranial

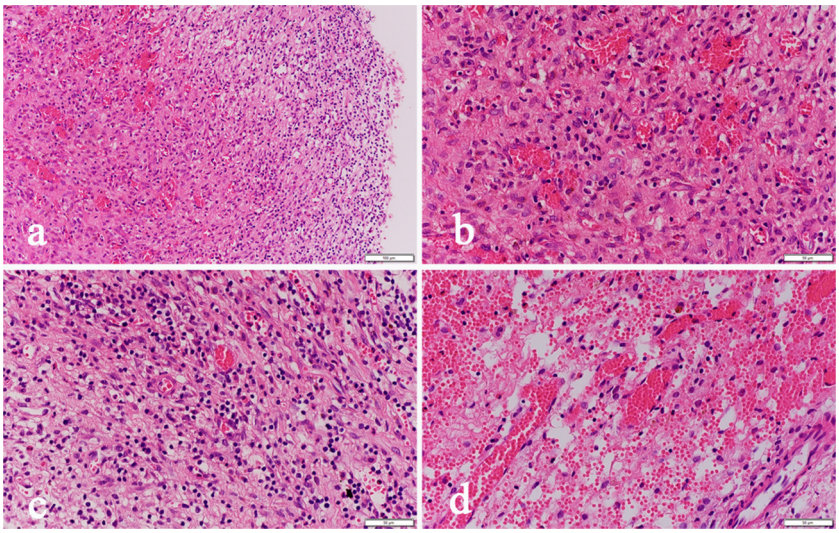

FIGURE 5 | Pathological sections of the hematoma capsule: **(a)** HE-02-20X shows visible hemorrhage, vascular hyperplasia and inflammatory cell infiltration inside the capsule. **(b)** HE-03-40X shows visible hemorrhage and fibrovascular hyperplasia inside the capsule. **(c)** HE-04-40X shows visible inflammatory cell infiltration. **(d)** HE-05-40X shows visible neovascular rupture and bleeding.

TABLE 4 | Multiple logistic regression analysis results of the risk factors associated with post-operative hematoma recurrence.

Risk factor	OR	95% CI	*p*-value
INET application (No)	Reference		
INET application (Yes)	3.71	1.31–9.62	0.02
Age>65 years	Reference		
Age≤65 years	1.51	1.05–2.87	0.03
Bilateral subdural hematoma	Reference		
Unilateral subdural hematoma	1.76	1.05–3.41	0.02

OR, odds ratio; CI, confidence interval; P-value of Wald test.

infection in the control group all exceeded 5 days. The low efficiency of simple BHD and the long duration of drainage were important causative factors of intracranial infection. Multiple logistic regression analysis showed that INET surgery (OR 3.71, 95% CI 1.31–9.62, $p = 0.02$), age of 65 years or younger (OR 1.51, 95% CI 1.05–2.87, $p = 0.03$) and unilateral subdural hematoma (OR 1.76, 95% CI 1.05–3.41, $p = 0.02$) were independent factors that reduced the post-operative recurrence rate (**Table 4**). Because the factors age and bleeding sites cannot be changed, our findings further show the value of INET in clinical application.

CONCLUSIONS

In summary, the growth and recurrence of subacute-chronic and chronic septal subdural hematomas are complicated processes. Our study suggests that the inflammatory response in the hematoma cavity, angiogenesis in the hematoma capsule, and local hyperactivation of fibrinolysis may be associated with the growth and recurrence of subdural hematoma. Therefore, post-operative targeted drug treatments, such as glucocorticoids, anti-angiogenic drugs, and anti-fibrinolytic drugs, may improve the prognosis of patients, but this conclusion requires confirmation by further clinical studies. At present, surgery is the key treatment for this type of disease. The INET used in this study can be freely switched between water and air environments, making full use of the advantages of the two surgical environments. Surgical instruments can be delivered through the 3.7 mm working channel of the endoscope, which makes the operation more convenient and faster and the localization of the bleeding sites more accurate, thus reducing the probability of accidental injury to brain tissue when placing the instruments. Compared to conventional methods, the INET treatment plan, which is designed based on the structure of the hematoma sac wall and hematoma fluid composition, has a low hematoma recurrence rate and high safety and can improve patient prognosis, making it an effective surgical method for the treatment of this type of disease.

LIMITATIONS

The present study does have some limitations. First, it was a non-randomized concurrent control study conducted in two centers and this was liable to selection bias. Second, although strict inclusion/exclusion criteria were setted and statistical methods were used to control selection bias, we still couldn't avoid the occurrence of it. Third, the surgeons responsible for surgery are trained doctors, but differences in individual clinical skills are unavoidable. Therefore, the conclusions require confirmation in future multicenter randomized controlled trial and long-term follow up.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Shenzhen People's Hospital (LL-KT-2018245) and Nanfang hospital (NFEC-2015-034). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YZ, BD, YP, and AS conceived and designed the whole experiments and manuscript. YZ, JH, JX, and XZ performed the statistical analysis and interpreted the data. JL and PL contributed to the literature research. HW and WL acquired the data. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.01408/full#supplementary-material

Video 1 | After clearing the old blood in the hematoma cavity and confirmation of hemostasis, the visceral capsule of the hematoma cavity wall is removed by INET using the grasping forceps under the water environment; the resection range is larger than 5 cm.

Video 2 | After opening the visceral layer of the hematoma capsule, INET was used to enter the hematoma visceral capsule and the arachnoid space for exploration in the water environment. The septum is easily peeled off with only a little adhesion band remaining.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neurology

CASE REPORT
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A Clinicopathological Report of a 93-Year-Old Former Street Boxer With Coexistence of Chronic Traumatic Encephalopathy, Alzheimer's Disease, Dementia With Lewy Bodies, and Hippocampal Sclerosis With TDP-43 Pathology

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Chronic traumatic encephalopathy (CTE) was recently recognized as a new tauopathy in which multifocal perivascular phosphorylated tau aggregates accumulate in neurons, astrocytes, and neurites at the depths of the cortical sulci. Traumatic brain injury (TBI) in early or mid-life is known to be associated with an increased risk of dementia in late life. This case report describes a 93-year-old former street boxer with a premortem diagnosis of severe dementia, who showed pathological evidence of the coexistence of Alzheimer's disease, CTE, dementia with Lewy bodies, and hippocampal sclerosis with TDP-43 pathology. These findings suggest that TBI may trigger a variety of misfolded proteins leading to dementia. Currently, clear clinical diagnostic criteria for CTE have not been established. Therefore, clinicians should be aware that TBI is a risk factor for dementia and that CTE can overlap with other neurodegenerative diseases.

Keywords: Alzheimer's disease, chronic traumatic encephalopathy, dementia, dementia with Lewy bodies, phosphorylated tau, traumatic brain injury, TDP-43 pathology, tauopathy

INTRODUCTION

Traumatic brain injury (TBI) was long recognized as a risk factor for dementia (1–5). Chronic traumatic encephalopathy (CTE) refers to the neuropathological changes resulting from repeated episodes of TBI (3, 5–7). In the early twentieth century, the terms "punch-drunk" or "dementia pugilistica" were used to describe the clinical features of a distinct neuropsychiatric syndrome that affected boxers. In 1949, the term CTE was used as synonymous with "punch-drunk" to describe the neurological deficits resulting from repeated blows to the head (8). Later, there were additional reports describing the neuropathologic features of this condition (9). In 2016, the first NINDS/NIBIB consensus meeting defined the neuropathological criteria of CTE and confirmed that the pathognomonic lesions in CTE are accumulations of abnormally hyperphosphorylated

tau (p-tau) in astrocytes and neurons located around small blood vessels and at the depths of the cortical sulci (10). CTE, a new neurodegenerative tauopathy, was reported in athletes who played soccer, baseball, ice hockey, and rugby, as well as in military personnel exposed to explosive blasts (5–7, 11). Clinical presentation of CTE was divided into three phases: behavioral/psychiatric, cognitive, and motor (12, 13). Late/older onset cases present predominantly with cognitive impairment (14).

While CTE can be suspected clinically, at present, the definitive diagnosis of CTE can only be made following neuropathological examination of the brain (11, 15–17). CTE pathology contributes to the clinical presentation, and its interaction with comorbid neurodegenerative pathologies is unclear (9, 13, 18). This case report describes a late-life dementia case with a clinical history of TBI. Pathological examination of the brain showed CTE, with the coexistence of Alzheimer's disease (AD), dementia with Lewy bodies (DLB), and hippocampal sclerosis with TDP-43 pathology.

CASE REPORT

Clinical Presentation

This patient was enrolled in the Rush Memory and Aging Project, a longitudinal study of aging and dementia, which was approved by the Institutional Review Board of Rush University Medical Center. A signed, informed consent was obtained for annual clinical evaluations and a signed Anatomical Gift Act for brain donation. The annual clinical evaluations were uniform and structured with a medical history questionnaire, neurologic examination, and detailed cognitive testing. Diagnosis of dementia followed a multi-step procedure as described previously (19, 20).

The deceased subject was a 93-year-old right-handed male, who was a street boxer in his 20s with no history of loss of consciousness. He first developed memory problems at the age of 82. Initially, he seemed more forgetful, although he was still able to live independently. However, at the age of 83, he started having difficulties in daily living, such as managing his own finances, managing his own calendar, and he became less social. He had trouble learning new information. He was not depressed; however, he was more irritable. His Mini-Mental State Exam (MMSE) score was 24/30. Magnetic resonance imaging of the head showed cortical atrophy, enlarged ventricles with mild enlargement of the cavum septum pellucidum. The clinical diagnosis was a major cognitive disorder due to AD. Although he was treated with cholinesterase inhibitors, there was rapid cognitive decline, and his MMSE dropped to 0/30 several years after the diagnosis of AD. There was no family history of dementia. He died from multi-organ failure at the age of 93.

METHODS

Autopsy was performed 8.5 h postmortem. The brain weight was 1,083 g with moderate, diffuse cortical atrophy. Blocks dissected from the brain included midfrontal, midtemporal, inferior parietal, occipital, anterior cingulate, and entorhinal cortices with amygdala, mid-hippocampus, basal ganglia (at the level of the anterior commissure), anterior thalamus, midbrain (at the level of the exiting third nerve fibers), and the cerebellum, which included the dentate nucleus. Blocks were processed using standard techniques, and paraffin-embedded sections (6 μm) stained with hematoxylin–eosin (HE) were used to detect microinfarcts and evaluate arteriolosclerosis and hippocampal sclerosis (HS) as described previously (21). A modified Bielschowsky stain was performed to demonstrate diffuse and neuritic plaques and neurofibrillary tangles (NFTs), which were quantitated in five brain regions (midfrontal, midtemporal, inferior parietal, and entorhinal cortices and hippocampus) that had the highest density of these structures as described previously (19, 20). Immunohistochemistry was performed to localize phosphorylated tau (p-tau; AT8), α-synuclein, β-amyloid, and phosphorylated transactive response DNA-binding protein 43 kDa (pTDP-43) using methods described previously (19, 21).

RESULTS

Microscopic examination (**Table 1**) showed.

CTE Tauopathy

The NINDS/NIBIB Consensus criteria were used for the diagnosis and staging of CTE (10, 22). Findings required for the diagnosis of CTE were present and consisted of multiple perivascular foci of p-tau-positive aggregates in neurons, astrocytes, and neurites in all neocortical areas examined (bilateral middle frontal, middle temporal, and inferior parietal

TABLE 1 | Summary of brain pathologies.

Pathological diagnoses	Protein marker	Pathological changes
CTE—primary features	p-Tau	Multifocal aggregates in a perivascular location and at depths of sulci
CTE—Supportive features	p-Tau	Severe changes in CA3, CA4, CA2, CA1, and subiculum of hippocampus
		Present in basal ganglia, amygdala, and substantia nigra
Alzheimer's disease		
Thal score 5	β-Amyloid	Based on distribution of β-amyloid plaques
Braak score 5	Tau	Based on neurofibrillary tangle scores
CERAD—probable AD	Tau	Based on neuritic plaque scores and history of dementia
NIA-Reagan—intermediate likelihood of AD	Tau	Based on CERAD and neurofibrillary tangle scores
DLB, neocortical type	α-Synuclein	LBs in substantia nigra, limbic sites, and neocortices
TDP-43 pathology	TDP-43	Present in amygdala, entorhinal cortex, hippocampus, and anterior temporal pole cortex

CTE, chronic traumatic encephalopathy; p-tau, phosphorylated tau; CERAD, Consortium to establish a registry for Alzheimer's disease; DLB, dementia with Lewy bodies; TDP-43, transactive response DNA-binding protein 43 kDa.

cortices). The p-tau accumulation was mainly concentrated at the sulcal depths (**Figures 1A–C**). In the hippocampus, all sectors showed p-tau pathology, which was particularly prominent in CA1 sector (**Figure 1D**). This pattern of NFTs in the hippocampus differs from that observed in AD. According to the Consensus criteria, the pattern of hippocampal involvement was consistent with the coexistence of CTE and AD (10, 18, 22). Abnormal p-tau immunoreactive neuronal and astrocytic aggregates were also present in the basal ganglia, amygdala, raphe nuclei, and substantia nigra (**Figure 1E**). The characteristic pathologic features of multiple foci of p-tau distribution throughout the neocortex, subcortical nucleus, brain stem, cerebellum, and hippocampus are consistent with a diagnosis of stage IV CTE.

AD Pathology

β-Amyloid immunostaining showed widespread multifocal areas of Aβ deposits in the neocortex (**Figure 1F**), hippocampus, basal ganglia, cerebellum, and brainstem resulting in a Thal score of 5. In the Bielschowsky stained sections, quantitation of neuritic plaques (**Figure 1G**) along with the history of dementia provided a Consortium to establish a registry for Alzheimer's disease (CERAD) diagnosis of probable AD. Quantitation of NFTs showed a high density in the hippocampus (**Figures 1H,I**), which along with NFTs in the neocortical areas gave a Braak score of 5. A pathological diagnosis of high likelihood Alzheimer's disease was made using the NIA-Reagan diagnostic criteria (20). However, the unusually high density of NFTs predominantly in the CA1 of the hippocampus was consistent with a diagnosis of the coexistence of AD and CTE.

DLB, Neocortical Type

On α-synuclein immunostaining, LBs were identified in six different brain regions (midfrontal, mid-temporal, entorhinal and cingulate cortices, amygdala, and substantia nigral; **Figures 2A,B**). Lewy bodies in the substantia nigra were associated with mild neuronal loss and gliosis. Since LBs were present in the neocortex, substantia nigra, and limbic sites, this case met the pathologic criteria of DLB, neocortical type.

Hippocampal Sclerosis With TDP-43 Pathology

Coronal slabs of the brain showed moderate enlargement of the frontal and temporal horns of the lateral ventricles associated with severe hippocampal atrophy (**Figure 2C**), and this was confirmed by microscopy of the HE-stained section, which showed severe neuronal loss and gliosis in the CA1 of the hippocampus compatible with a diagnosis of hippocampal sclerosis (**Figure 2D**). Cytoplasmic TDP-43-positive inclusions were found in the neurons and glia in the amygdala (**Figure 2E**), entorhinal cortex, CA1 sector, and the dentate neurons (**Figure 2F**) of the hippocampus and in the anterior temporal pole cortex. The degree of non-p-tau-related pathology in the hippocampus was greater than that described in the CTE cases (10).

DISCUSSION

This case report describes the clinical and pathological findings of a previous street boxer who developed severe dementia (MMSE = 0). Brain examination showed the coexistence of AD, CTE, DLB, neocortical type, and hippocampal sclerosis with TDP-43 pathology. The finding of five pathologies in a single case is rare.

Clinically, CTE is reported to have an early or late onset. The early/younger age onset subtype is characterized by behavior and mood symptoms but with minimal cognitive or motor impairment, while the late/older onset subtype is characterized by cognitive impairment (14). There is significant correlation between the pathological stage of CTE and the duration of TBI exposure and the age at death (22). Clinically, it is difficult to distinguish the CTE subtypes because comorbid pathologies are common in CTE and due to an overlap with symptoms of other neurological diseases (14, 16, 23). The subject in the present study had the late onset subtype of CTE. A recent report suggested that cognitive reserve may mitigate cognitive decline in older individuals with early life TBI (14). However, once cognitive decline occurs, cognitive deterioration is severe, possibly due to the contribution of comorbid pathology as in the present case. Dementia is reported to be associated with DLB, neocortical type, and the stage of CTE in addition to AD pathology and age at death (23). An early or mid-life TBI as a risk factor for dementia in late life is well-recognized (2, 3, 24).

There are reports of CTE cases with coexisting additional pathologies. In a group of 68 cases with a pathological diagnosis of CTE and a history of antemortem TBI, 85% had coexisting TDP-43 pathology, 11% had coexisting Alzheimer's disease, 16% had coexisting Lewy body disease, while 6% had coexisting frontotemporal lobar degeneration (25). In a group of six football players with TBI and progressive cognitive impairment, postmortem examination showed that AD and TDP-43 often coexist with CTE (5). In another study (18), of eight soccer and rugby players with dementia and a pathological diagnosis of CTE cases, there was coexistence of AD pathology in seven cases, of TDP-43 pathology in six cases, and one case showed CTE associated with DLB pathology, while in five cases, there was coexistence of three pathologies (CTE, AD, and TDP-43). None of the reported cases showed the coexistence of five pathologies in a single case as observed in the present case. The coexistence of multiple pathologies suggests that TBI may initiate or accelerate multiple proteinopathies resulting in several different neurodegenerative processes (3, 5) resulting in dementia (2–5). One of the mechanisms that results in the development of dementia after TBI is AD-related β-amyloid and tau pathology (26). However, how TBI triggers multiple proteinopathies remains uncertain.

It is well-established that p-tau is a biomarker for CTE. Although there are different tauopathies, the pathognomonic lesion of CTE, consisting of multifocal, perivascular p-tau aggregates in neurons, astrocytes, and neurites at the depths of the cortical sulci is distinct and not found in the other tauopathies (10). However, the neuronal p-tau found in CTE does share a similar profile regarding isoform ratio and phosphorylation state as the tau in AD (10). Both CTE and

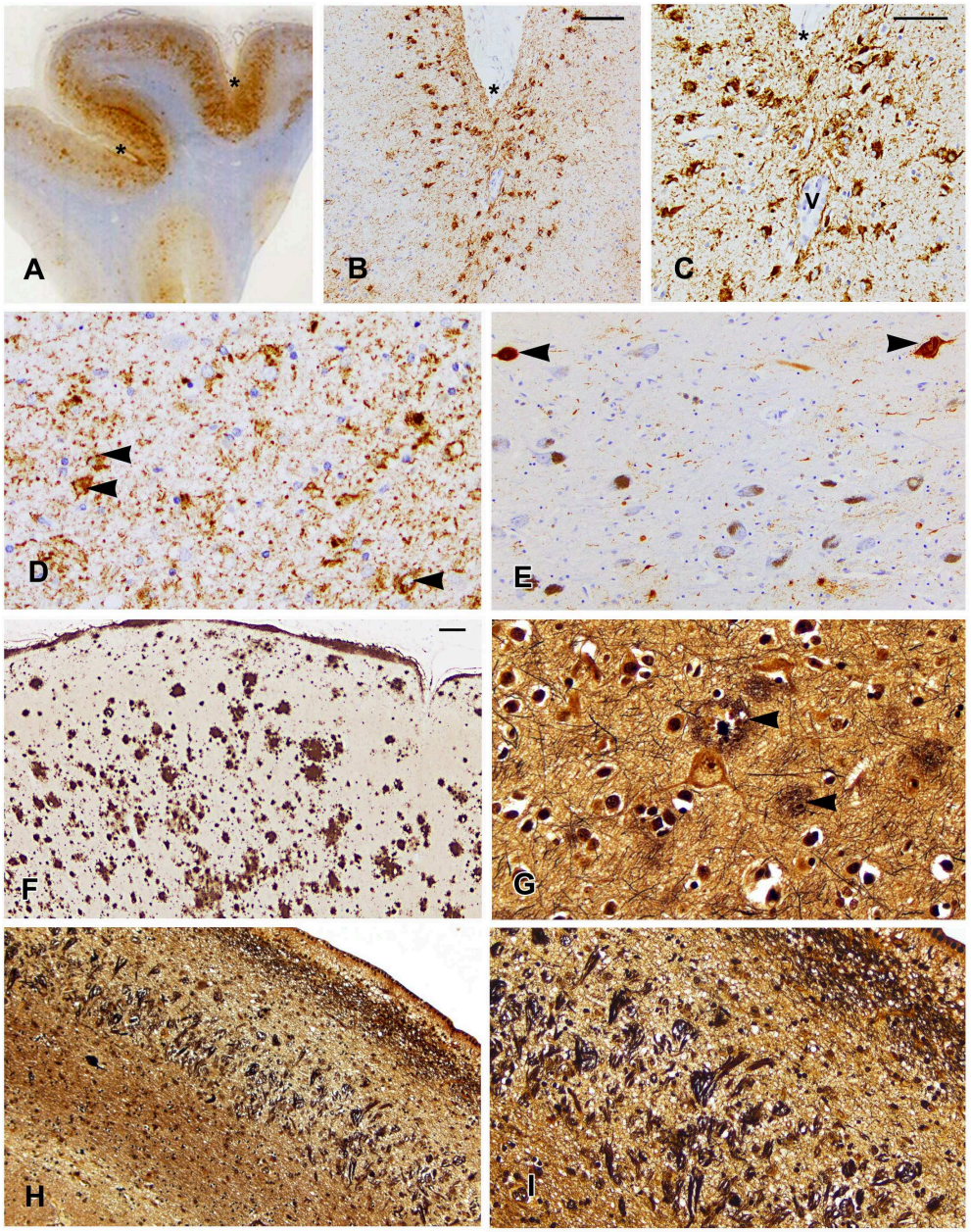

FIGURE 1 | Localization of p-tau **(A–E)**, β-amyloid **(F)**, and tau in Bielschowsky-stained sections **(G–I)** are shown. **(A)** A whole mount of the parietal cortex shows increased p-tau immunoreactivity in the cortex at the depths of sulci, which are marked by asterisks in **(A–C)**. **(B)** Note the concentration of p-tau-positive neurons in the cortex at the depth of the sulcus **(B,C)** and surrounding a vessel marked by "V" **(C)**. **(D)** The CA1 sector of the hippocampus shows p-tau immunostaining in residual neurons (arrowheads) and in neurites. **(E)** The substantia nigra shows loss of neurons and tau immunostaining in two neurons (arrowheads). **(F)** Low magnification of the midfrontal cortex shows the extent of β-amyloid deposition. **(G)** The midfrontal cortex shows neuritic plaques (arrowheads). **(H)** A low power photomicrograph of the CA1 sector of the hippocampus shows dense collections of neurofibrillary tangles and ghost tangles, which are shown in higher magnification in **(I)**. Scale bars: **(B,F,H)** = 100 μm; **(C–E,G,I)** = 50 μm.

AD share the characteristic feature of hyperphosphorylated tau, where phosphorylation at specific residues occurs as an ordered process, leading to tau aggregation and oligomer formation (6). However, in the present case, the severity of CA1-predominant neurofibrillary degeneration in the hippocampus supports the coexistence of AD and CTE (10, 22). Nevertheless, the differential diagnosis of hippocampal p-tau pathology in CTE and AD requires further study (10). Aβ plaques, especially diffuse Aβ plaques, are present in some cases of CTE, but they are not a consistent feature of CTE and represent the coexistence of AD (10). Aβ deposition is reported to occur at an earlier age and at an accelerated rate and is

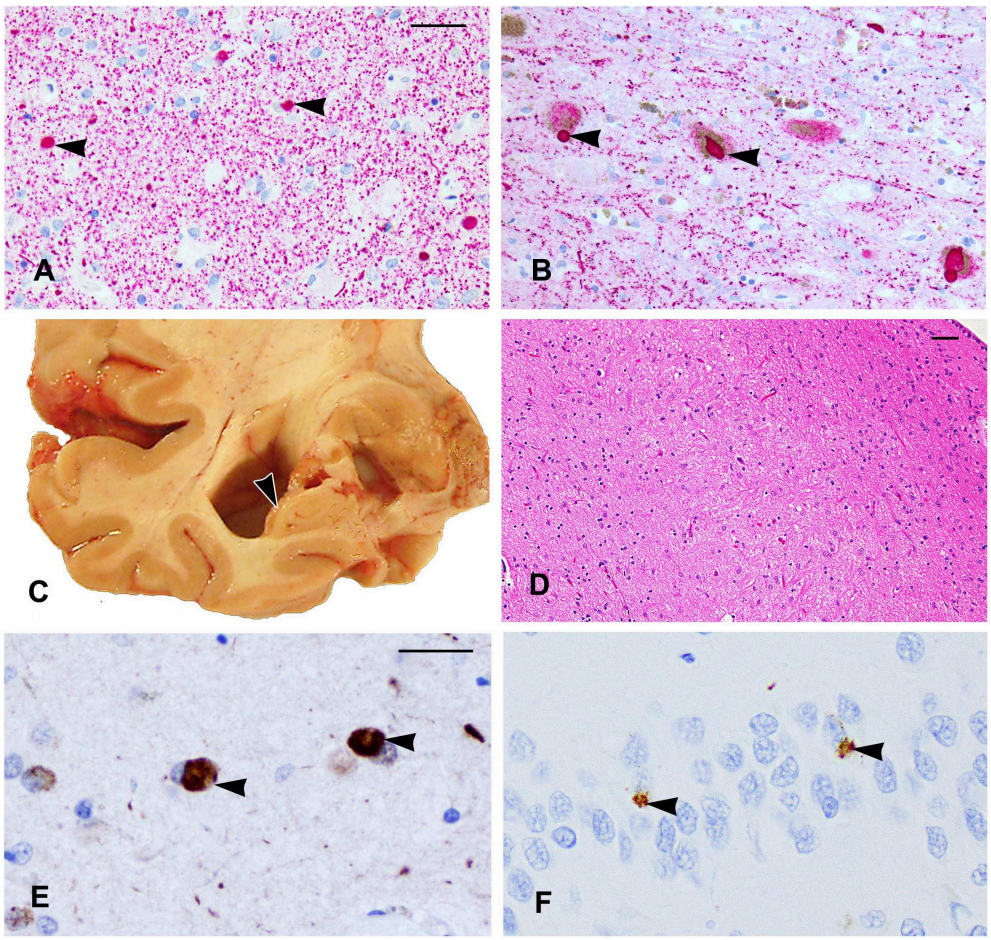

FIGURE 2 | (A,B) α-Synuclein immunostaining shows Lewy bodies in the amygdala **(A)** and in the substantia nigra (arrowheads). **(C)** The right temporal lobe showing severe atrophy of the hippocampus (arrowhead) with enlargement of the adjacent inferior horn of the lateral ventricle. **(D)** A segment of the CA1 sector of the hippocampus shows few residual neurons and mainly glial nuclei due to hippocampal sclerosis. **(E,F)** TDP-43 cytoplasmic inclusions are shown in the amygdala **(E)** and neurons of the dentate gyrus **(F)** (arrowheads). Scale bars: **(A,B)** = 50 μm; **(D)** = 100 μm; **(E,F)** = 25 μm.

associated with increased clinical and pathological severity in CTE (23).

Investigation of the clinical and pathological relationships between CTE and DLB in a group of deceased athletes reported that CTE significantly increased the odds of having DLB, neocortical type (23). A study based on community-dwelling older persons confirmed that DLB, neocortical type plays an independent role in cognitive impairment and has a deleterious effect on many aspects of cognition in older persons (27, 28). DLB, neocortical type provides an adequate explanation for cognitive impairment or dementia (27, 28), and it lowers both the level of cognitive function and increases the pace of cognitive dysfunction in persons with AD pathology. The additional effect of LB pathology appears to be highly deleterious, lowering global cognition by a full standard deviation while increasing the odds of dementia by 43-fold (28).

The association of TDP-43 pathology with hippocampal sclerosis is well-documented, and hippocampal sclerosis is reported to be more common in those aged >90 years (29). The distribution of TDP-43 pathology in the present case is greater than that described in CTE (10). TDP-43 pathology was reported in nearly half of the older community-dwelling persons (30). A previous study reported that TDP-43 pathology starts from the amygdala before spreading to the hippocampus and other brain regions in aging and AD (21). In the present case, since TDP-43 pathology extended to the anterior temporal pole cortex, this pathology could contribute to dementia since extension of TDP-43 pathology to the anterior pole cortex was reported to be associated with increased odds of dementia and impaired episodic memory (21). Contributing to dementia in this case is the finding of hippocampal sclerosis, which, with coexisting TDP-43 pathology, is associated with lower function in multiple cognitive domains (29). Further study is necessary to clarify the combined roles of CTE and hippocampal sclerosis with TDP-43 pathology in dementia.

Currently, an estimated 1.6–3.8 million concussions occur annually in the US, with American football, hockey, soccer, and

lacrosse accounting for most of the sports-related concussions (31). Head impact in contact sports or in warfare that do not result in apparent clinical symptoms may still result in neuronal injury and late onset CTE and other neurodegenerative diseases including DLB and AD (9, 32). Additional research is necessary to determine the contribution of p-tau and other pathologies to the development of the clinical symptoms of CTE. Early diagnosis and effective intervention could be a key strategy to prevent TBI-induced CTE and the associated neurodegenerative diseases that lead to loss of brain function and cognitive deficits.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Rush University Medical Center. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Endocannabinoid Modulation of Microglial Phenotypes in Neuropathology

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Microglia, the resident immune cells of the central nervous system, mediate brain homeostasis by controlling neuronal proliferation/differentiation and synaptic activity. In response to external signals from neuropathological conditions, homeostatic (M0) microglia can adopt one of two activation states: the classical (M1) activation state, which secretes mediators of the proinflammatory response, and the alternative (M2) activation state, which presumably mediates the resolution of neuroinflammation and tissue repair/remodeling. Since chronic inflammatory activation of microglia is correlated with several neurodegenerative diseases, functional modulation of microglial phenotypes has been considered as a potential therapeutic strategy. The endocannabinoid (eCB) system, composed of cannabinoid receptors and ligands and their metabolic/biosynthetic enzymes, has been shown to activate anti-inflammatory signaling pathways that modulate immune cell functions. Growing evidence has demonstrated that endogenous, synthetic, and plant-derived eCB agonists possess therapeutic effects on several neuropathologies; however, the molecular mechanisms that mediate the anti-inflammatory effects have not yet been identified. Over the last decade, it has been revealed that the eCB system modulates microglial activation and population. In this review, we thoroughly examine recent studies on microglial phenotype modulation by eCB in neuroinflammatory and neurodegenerative disease conditions. We hypothesize that cannabinoid 2 receptor (CB2R) signaling shifts the balance of expression between neuroinflammatory (M1-type) genes, neuroprotective (M2-type) genes, and homeostatic (M0-type) genes toward the latter two gene expressions, by which microglia acquire therapeutic functionality.

Keywords: neuroinflammation, immunomodulation, microglia subtype, alternative activation, M0/M1/M2 polarization, endocannabinoids, CB2 receptor agonist, animal disease model

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INTRODUCTION

In the last several decades, a growing body of evidence has revealed an intricate cross talk between neurons and immune cells to maintain brain homeostasis (1, 2). If this delicate equilibrium is disrupted by any pathological stimuli, the inflammatory response can be exaggerated in the central nervous system (CNS). In response to neuroinflammation, microglia, the resident macrophages of the CNS, undergo morphological, phenotypic, and functional changes. Evidence has shown that upon activation microglia can cause deleterious effects on neuronal cells by releasing reactive

oxygen and nitrogen species, cytokines, chemokines, and other inflammatory mediators. The dying neurons, in turn, release more stimulatory factors, which exaggerate the activation of microglia. This vicious cycle contributes to the pathogenesis of neurodegenerative diseases. On the other hand, several recent studies have shown that under certain experimental settings microglia, similar to macrophages in the periphery, display an alternative activation state that presumably leads to cytoprotective effects by secreting trophic factors and tissue remodeling molecules. Moreover, microglia *in vivo* have been observed to display characteristics that resemble the alternative activation state, which is designated as the M2 state as opposed to the classical activation M1 state.

Microglia/macrophages in the alternative activation state are believed to be critically involved in neuronal cell repair, tissue remodeling, including debris clearance, and the resolution of inflammation (3). Thus, in order to halt the vicious cycle of neuroinflammation and prevent neuronal injury, it is crucial to control or modulate microglial activation states rather than eliminate microglial activity (4, 5). Over the past decade, the neuroprotective effects of endocannabinoids (eCB) have received a significant amount of attention. Numerous studies have shown that activation of eCB signaling can suppress microglial activation and ameliorate neurodegeneration in several neurological diseases. The therapeutic mechanisms of eCB signaling are at least partially due to the modulation of microglial polarization. In this review, we summarize recent studies, mainly published in the last decade, regarding the regulation of microglial polarization by the eCB system in both *in vitro* cell cultures and disease animal models. We propose that cannabinoid type 2 receptor (CB2R)-mediated signaling plays a vital role in the modulation of microglial polarization, and we evaluate some issues that should be addressed. Although we briefly outline the eCB system in the CNS and microglial activation hereafter, several excellent and comprehensive review articles regarding the eCB system (6–9) and microglial/macrophage polarization (10–13) are available; readers are encouraged to review these articles to understand the related topics.

Abbreviations: AA-5HT, N-arachidonoylserotonin; Aβ, amyloid beta; Abhd 6/12, α-β-hydrolase domain 6/12; AD, Alzheimer's disease; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; ALS, amyotrophic lateral sclerosis; APP/PS1, amyloid beta precursor protein/presenillin1; Arg1, arginase 1; Bax, Bcl2 associated X; BBB, blood brain barrier; Bcl2, B-cell lymphoma 2; BCP, beta-caryophyllene; Bdnf, brain-derived neurotrophic factor; BrdU, bromodeoxyuridine; Casp3, Caspase 3; CB1/2R, cannabinoid 1/2 receptor; CCI, controlled cortical impact; Ccr, C-C motif chemokine receptor; Ccl, C-C motif chemokine ligand; CD, cluster of differentiation; CNS, central nervous system; COX IV, cytochrome *c* oxidase subunit 4; Cox2, cyclooxygenase2; Creb, cAMP response element binding protein; Cx3cr1, CX3C receptor 1; Cxcl, C-X-C motif chemokine ligand; CYP450, cytochrome P450; Daglα/ β, diacylglycerol lipase α/β; DAMPs, Danger Associated Molecular Patterns; DAT, dopamine active transporter; Dbcn, doublecortin; dpi, days post infection; EAE, experimental autoimmune encephalopathy; eCB, endocannabinoid; EEQ-EA, epoxyeicosatetraenoic ethanolamide; EDP-EA, epoxydocosapentaenoic ethanolamide; ERK, extracellular signal-regulated protein kinase; Faah, fatty acid amide hydrolase; Fizz1, found in inflammatory zone 1; Gdnf, glial cell-derived neurotrophic factor; Gfap, glial fibrillary acidic protein; GMH, germinal matrix hemorrhage; GPR, G protein-coupled receptor; HSP, heat shock protein; Iba1, ionized calcium binding adaptor molecule 1; Icam1, intercellular adhesion molecule 1; Igf1, insulin-like growth factor 1; Ifnγ, interferon γ; IL, interleukin; IκBα, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α; iNOS, inducible nitric oxide synthase; JNK, c-Jun NH2 terminal kinase; lamp1, lysosomal-associated membrane protein 1; LPS, lipopolysaccharide; Mac1, macrophage-1 antigen; Magl, monoacylglycerol lipase; MAPK, mitogen-activated protein kinase; Mbp, myelin basic protein; MCAO, middle cerebral artery occlusion; Mcp-1, Ccl2; M-MDSC, monocytic-myeloid derived suppressor cells; MHCII, major histocompatibility complex II; Mip-1α/β, macrophage inflammatory protein-1 α/β; Mmp9, matrix metallopeptidase 9; Mog, myelin oligodendrocyte glycoprotein; Mpo, myeloperoxidase; MS, multiple sclerosis; Msr1, macrophage scavenger receptor 1; mtDNA, mitochondrial deoxyribonucleic acid; Nape-pld, N-acyl phosphatidyl ethanolamine phospholipase D; NF-κB, nuclear factor κ light-chain-enhancer of activated B cells; Ngf, nerve growth factor; NK cells, natural killer cells; NLR, NOD-like receptor; Nrf1, nuclear respiratory factor 1; NT3, neurotrophin 3; OPC, oligodendrocyte precursor cell; PAMPs, Pathogen Associated Molecular Patterns; PD, Parkinson's disease; Pgc-1α, Pparγ coactivator 1-α; PGE2, prostaglandin E2; Plp, proteolipid protein; PMCAO, permanent middle cerebral artery occlusion; PNS, peripheral nervous system; Ppar, peroxisome proliferator-activated receptor; PRR, pattern recognition receptor; Rantes, Ccl5; ROS, reactive oxygen species; Socs3, suppressor of cytokine signaling 3; SRs, scavenger receptors; TBI, traumatic brain injury; Tarc, Ccl17; T-bet, T-box–containing protein expressed in T cells; Tfam, mitochondrial transcription factor A; Tgfβ, transforming growth factor β; TH, tyrosine hydroxylase; Th1, T helper type 1; TLRs, Toll-like receptors; TMEV, Theiler's Murine Encephalomyelitis Virus; Tnfα, tumor necrosis factor α; Trem2, triggering receptor expressed on myeloid cells 2; VD, vascular dementia; Ym1, chitinase-3-like protein 3.

KEY PHARMACOLOGICAL ECB COMPONENTS IN THE CNS

The cannabinoid type 1 receptor (CB1R) was first cloned as the binding receptor for Δ^9-tetrahydrocannabinol, the main psychologically active compound in *Cannabis sativa* (14), and CB2R was later cloned in 1993 (15). Since then, a variety of plant-derived and synthetic compounds that target cannabinoid (CB) receptors have been identified and developed as agonists or antagonists. In parallel, endogenous CB ligands were also discovered; anandamide (AEA), which was discovered in 1992 (16), and 2-arachidonoyl glycerol (2-AG), discovered in 1995 (17, 18), are the best-characterized eCB ligands. AEA binds to both CB receptors as a partial agonist, while 2-AG binds to these receptors as a full agonist (19–21). Later on, several new components of the eCB system, including ethanolamine, glycerol, or amino acid derivatives of acyl fatty acids, such as N-palmitoylethanolamine, 2-oleoylglycerol, and N-arachidonoylglycine, were identified in the CNS and shown to be involved in eCB signaling.

CB1R is one of the most abundantly expressed G-protein coupled receptors in the CNS and is primarily expressed in neurons. CB1R is localized in presynaptic terminals where its activation negatively modulates neurotransmission. Thus, CB1R signaling is the critical neuronal regulator for the control of motor function, emotion, cognition, memory, and analgesia (22). CB2R is highly expressed in immune cells, such as B cells, NK cells, and macrophages, in the peripheral nervous system (PNS) and predominantly in microglia in the CNS. Moreover, since CB2R expression is upregulated in tissues under pathological stimuli (23), CB2R is regarded as the central component of the eCB system involving the inflammatory response. With regard to downstream signaling, both CB1R and CB2R have

two independent pathways: the canonical G-protein-dependent pathway and the non-canonical G-protein-independent pathway. Upon ligand binding, adenylyl cyclase is inhibited by the activation of $G_{i/o}$, the main G protein subunit associated with CBs. As a result, cAMP is reduced, followed by modulation of its downstream signal transducers, such as protein kinase A. CBs are also associated with $G_{\beta\gamma}$ proteins, which initiate other signaling pathways that activate certain calcium and potassium ion channels and several mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated protein kinase (ERK), c-Jun NH2 terminal kinase (JNK), and p38 MAPK pathways (24). These pathways are involved in cell proliferation, migration, and cytokine production. Additionally, non-canonical CB signaling can be mediated by β-arrestin (25). β-arrestin is the scaffold protein associated with CBs, and it regulates their internalization and desensitization. β-arrestin and several other signal molecules are recruited to form complexes with CB receptors to act as either receptor signal transducers or terminators (26). Thus, CBs mediate multiple signaling pathways that intricately cross talk with each other. The output signaling is impacted by surrounding microenvironments and intracellular conditions. In addition, the selectivity and preference of downstream CB signaling is determined by the CB ligands, endogenous AEA and 2-AG, or the synthetic CB agonists (27, 28). Considering these CB ligands may also have off-target effects (29), regulation of this complex signaling system by eCB modulation has not yet been completely elucidated.

After the discovery of the endogenous ligands, several enzymes responsible for their biosynthesis and metabolism in the CNS were identified. The major synthesizing enzymes for 2-AG are diacylglycerol lipase (DAGL)α and DAGLβ (30), by which diacylglycerol is converted to 2-AG. DAGLα is the major biosynthesizing enzyme in neurons, while DAGLβ is the major biosynthesizing enzyme in microglia (31). There are multiple pathways responsible for the biosynthesis of AEA; N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), which catalyzes the cleavage of N-acylethanolamine from N-arachidonoyl-phosphatidylethanolamine, is considered the main biosynthetic enzyme (32). 2-AG degradation occurs mainly through monoacylglycerol lipase (MAGL) (33, 34) but also through α-β-hydrolase domain (ABHD)6 (35) and ABHD12 (36) to a lesser extent (37). The chief degrading enzyme of AEA is fatty acid amide hydrolase (FAAH). In addition, eicosanoid biosynthetic enzymes such as cyclooxygeanase-2 (COX-2), lipoxygenase12/15, and cytochrome P450 (CYP450) are also involved in eCB metabolism due to the structural similarity between eCB ligands and the eicosanoid precursor, arachidonic acid (38). In order to boost eCB signaling, several enzyme inhibitors have been developed to block the activity of the eCB-degrading enzymes. The inhibitors of MAGL (i.e., JZL184), ABHD6 (i.e., WWL70), and FAAH (i.e., PF3845, URB597) have been extensively investigated with regard to their pharmacological efficacy *in vitro* and in *in vivo* disease models. **Figure 1** shows a schematic diagram of CB2R signaling pathways and eCB metabolic pathways, including enzyme inhibitors, in microglia.

As new eCB ligands were discovered, additional receptors were identified and coupled with eCB signaling and function. These receptors include transient receptor potential vanilloid 1 (TRPV1) (39), GPR55 (40), GPR18 (41), and peroxisome

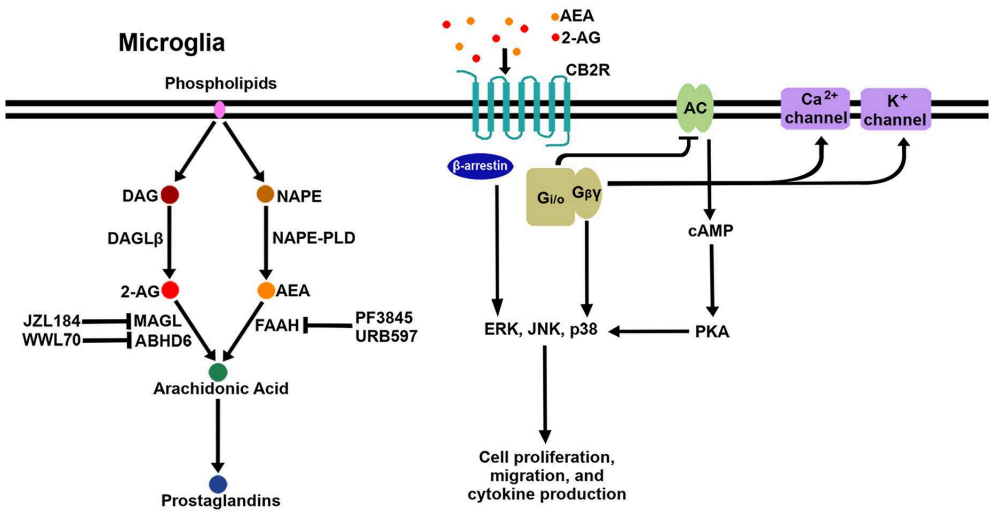

FIGURE 1 | Schematic signaling pathways and biosynthesis/degradation of endocannabinoids in microglia. When eCB bind to CB2R on the microglial cell surface, the receptor initiates signaling through the canonical G-protein-dependent pathway and the non-canonical G-protein-independent pathway. Adenylyl cyclase (AC) is inhibited by the activation of $G_{i/o}$ proteins; as a result, cAMP is reduced, followed by modulation of PKA signaling. $G_{\beta\gamma}$ proteins activate certain calcium and potassium ion channels. Additionally, β-arrestin is recruited to CB2R to act as a receptor signal transducer or terminator. Three arms of the CB2R signaling pathway activate multiple downstream pathways, including several MAPKs (ERK, JNK, and p38 MAPK). AEA and 2-AG are mainly biosynthesized from NAPE by NAPE-PLD and from DAG by DAGLβ, respectively. AEA is degraded to arachidonic acid by FAAH, which is inhibited by PF3845 and URB597. 2-AG is degraded to arachidonic acid by MAGL and ABHD6, which are inhibited by JZL184 and WWL70, respectively. Arachidonic acid is a key precursor for prostaglandins.

proliferator-activated receptors (PPARs) (42). PPARγ is activated when it binds to one of several lipid mediators, such as AEA and other N-acylethanolamines; it then acts as a transcription factor for a subset of genes that are involved in energy and lipid metabolism, oxidative stress inhibition, and the anti-inflammatory response (43). Furthermore, PPARγ has been recognized as a modulator of microglial alternative activation since treatment with a PPARγ agonist triggers alternative activation of microglia *in vitro* and in a chronic stress model (44).

FUNCTION OF MICROGLIA UNDER ACTIVATED CONDITIONS

Although there is still debate about macrophage/microglial ontogeny, microglia are currently believed to develop from early erythromyeloid progenitor cells that originate in the yolk sac and migrate to the CNS, whereas monocyte-derived macrophages develop from hematopoietic stem cells (45). Microglia have unique physiological functions in the CNS (46), including synaptic organization (47), trophic support for neurons (48), and regulation of neuronal excitability (49). Nevertheless, macrophages and microglia share many functions as sentinels and effectors of the immune response in the PNS and CNS, respectively. Upon brain injury, a substantial number of blood macrophages are activated and infiltrate the parenchyma. Since the immune response of infiltrated macrophages is quite similar to that of microglia, the immunological roles of microglia and macrophages are difficult to distinguish; nevertheless, these two cell types can generally be identified by the expression levels of cell surface marker CD45 ($CD45^{low}$ for microglia; $CD45^{high}$ for macrophage) or by specific markers for microglia, such as Tmem119 (50) and P2ry12 (51). Therefore, most of the experimental data for the immune response and phenotype characterization described in this review are thought to be influenced by both types of cells unless specified.

Initiation of the Microglial Immune Response

Regulation of microglial activation is mostly dependent on the interaction of microglia with molecules in the brain parenchyma. These extracellular molecules secreted from adjacent cells are recognized by a variety of different receptors expressed in the cytoplasmic membrane or cytoplasm; these receptors are known as Pattern Recognition Receptors (PRRs). The PRRs expressed in glial cells mainly consist of Toll-like receptors (TLRs), NOD-like receptors (NLRs), and scavenger receptors (SRs) (52). Each type of PRR binds to specific molecules, some of which are known as Pathogen Associated Molecular Patterns (PAMPs). PAMPs are molecules of exogenous origin and are associated with pathogens; PAMPs include bacterial membrane components, such as lipoprotein or peptidoglycan, and bacterial nucleic acid (unmethylated DNA or RNA) (53). On the other hand, certain types of PRRs can react with Danger Associated Molecular Patterns (DAMPs), which are of intracellular origin and are released to the extracellular space or other compartments after CNS injury (54). DAMPs include a variety of cellular components, such as proteins (Amyloid β, S100, heat shock proteins, thioredoxin, high-mobility group box 1), nucleic acids (mitochondrial DNA/RNA), and molecules from the extracellular matrix (hyaluronic acid, fibronectin) (55). In addition, small molecules like ATP and calcium ions can drive microglia to move toward the lesion site and trigger phenotypic change (56, 57). Considerable data show that PRRs are essential for surveillance of CNS homeostasis and are among the first responders to CNS injury. Both PAMPs and DAMPs directly induce proinflammatory cascades and the formation of the inflammasome, and therefore they mediate the release of cytokines (58). Microglial activation is, in turn, regulated by the cytokines or chemokines released from the immune cells at lesion sites in a paracrine and/or autocrine manner.

Microglial Classical Activation

Under physiological conditions, microglia maintain a ramified cell shape. However, in response to abnormal microenvironments and factors, microglia adopt a phagocytic phenotype, in which the small soma becomes enlarged, and the number and length of processes progressively decrease until the cell attains an amoeboid morphology (55, 59–61). As a first line of defense, the classical activation (M1) of microglia is geared toward killing pathogens or infected cells, and it subsequently triggers the antigen presentation response to induce the adaptive immune system. Reactive oxygen or nitrogen species are a powerful tool for destroying pathogens and infected cells. These molecular species are mainly derived from inducible nitric oxide synthase (iNOS), myeloperoxidase, and NADPH oxidase in reactive microglia. During classical activation, these enzymes are upregulated and activated, and, as a result, the production of reactive oxygen or nitrogen species is increased. Regarding the adaptive immune response, several of the associated receptors and enzymes are upregulated. For instance, major histocompatibility complex II (MHCII), CD86, and Fcγ receptors are upregulated in the classical activation state. These receptors are involved in the antigen-presenting activity of microglia and interact with T cells that have infiltrated the brain parenchyma (62).

Microglial Alternative Activation

After the onset of classical activation to eliminate pathogens, resolution of inflammation and restoration of brain homeostasis are required. The initial classical activation is followed by a secondary alternative activation (M2), which is important for wound healing and suppression of inflammation. The existence of two distinct phenotypes was first theorized based on the original finding that the IL-4-mediated inflammatory response adopts an alternative activation associated with a reduction in proinflammatory cytokines in macrophages (63). Subsequent studies demonstrated that the alternative (M2) phenotype was characterized by the augmented expression of anti-inflammatory cytokines (i.e., IL-4, IL-10, and IL-13), trophic factors, such as transforming growth factor-β (TGF-β) and insulin-like growth factor 1 (IGF1), and metabolic or tissue remodeling genes, such as arginase1 (Arg1) and chitinase-3-like protein 3 (Ym1) (64–69). Arg1 catalyzes L-arginine to urea and L-ornithine,

which is the precursor for polyamine biosynthesis and inhibits classical activation (M1) by competing with NO generation from iNOS. Furthermore, the alternative (M2) phenotype was found to be further classified into multiple subtypes based on different sets of cytokine expression and receptor profiles, similar to macrophages. Treatment with IL-4 and IL-13 induced the expression of SRs for phagocytosis and anti-inflammatory molecules, such as Ym1, Fizz1, and IGF1. The subtype induced by these cytokines, classified as M2a, is presumably important for the resolution of inflammation and the clearance of cell debris. The M2c subtype, which is induced by TGF-β, IL-10, and glucocorticoids *in vitro*, is characterized by a deactivating phenotype and postulated to be involved in tissue remodeling and matrix deposition (69). In macrophages there exists another alternative state, M2b, which is more closely related to the M1 phenotype; however, the M2b activation state is not clearly seen in microglia. Of note, current classification of microglia/macrophages into certain phenotypes is based on cell culture studies *in vitro*. The microglia/macrophages are stimulated by individual cytokines, such as IFNγ, IL-4, or TGF-β, and a subset of genes and the cell morphology are observed (70). **Figure 2** shows a schematic diagram of the microglial phenotypes and their typical gene markers.

However, in brain pathologies and even in physiological milieu (e.g. aging) (71), the mixture of cytokines, the variety of the surrounding matrix, and the different microenvironmental factors influence the polarization and gene expression of microglia/macrophages (12). In several studies using animal models, microglia and macrophage phenotypes have not been clearly defined and often have mixed profiles due to the environmental milieu in which both classical and alternative activation inducing cues are present (72). Therefore, the two phenotypes or markers of both phenotypes are sometimes observed simultaneously in the same cell (73). The typical phenotypes of the M1 and M2 states have not been proven *in vivo*. Some transcriptomic studies at the single-cell level have attempted to characterize microglial heterogeneity in disease animal models or pathological conditions (74–77); however, the studies have not identified microglial subset characteristics for the M1 or M2 polarized phenotypes. It was proposed that the M1/M2 phenotype is an oversimplification or even non-existent *in vivo* based on experimental evidence (78, 79). However, the terms M1 and M2 do not only indicate specific microglial subsets; they also indicate individual microglial metabolic and gene regulation states: the neuroinflammatory and neuroprotective states, respectively. We continue to use these terms in this review article since they are still useful for characterizing microglial states and for evaluating pharmacological efficacy in relation to microglial activity, as described in previous reports discussed below. Nevertheless, we will later discuss this discrepancy *in vivo* between single-cell transcriptomic and histopathological findings.

REGULATORY ROLE OF ECB ON MICROGLIAL POLARIZATION IN CELL CULTURE

The eCB system has long been recognized as a modulator of neuronal synaptic activity and the inflammatory response. Our knowledge on the role of eCB in the immune system has rapidly expanded in the last decade, and accumulating evidence shows that the eCB system is deeply involved in regulating polarization phenotypes in microglia. In this section, recent *in vitro* studies regarding microglial modulation by the eCB system are reviewed and summarized (**Table 1**).

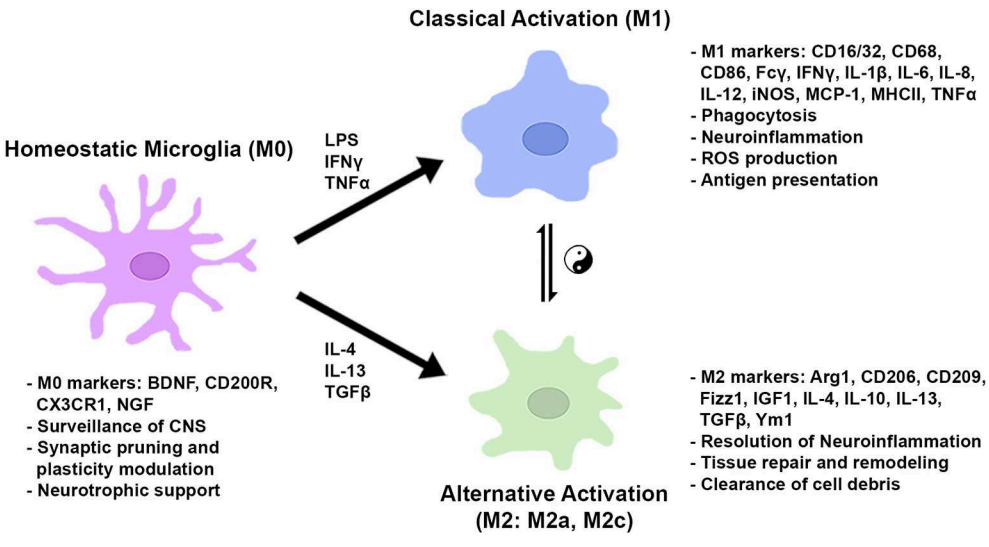

FIGURE 2 | Microglial metabolic and gene regulatory states based on *in vitro* studies. In normal conditions, microglia take on a homeostatic state expressing genes for CNS surveillance, synaptic modulation, and neuronal trophic support. In the presence of pro-inflammatory stimuli, microglia are classically activated to induce genes for phagocytosis, ROS production, and antigen presentation. Anti-inflammatory cytokines activate genes to resolve inflammation, repair/remodel tissue, and clear cell debris. Microglial cells can shift between gene regulatory states dependent on environmental cues and stimulatory conditions.

TABLE 1 | Effects of eCB modulation on microglial polarization *in vitro*.

Treatment	Cell culture	eCB activation method	M1/M2 phenotype	Other key findings/antagonist tests	References
LPS (1 μg/mL) 24 h	mouse primary MG	BCP (1 μM) 24 h prior to LPS	IL-1β/TNFα/PGE2/iNOS/NO/ROS ↓; IL-10/urea/Arg1/GSH ↑	cell proliferation up; AM630 (1 μM) but not GW9662 (1 μM) reversed	(80)
LPS (100 ng/mL) 8–24 h	BV2	PF3845 (10 μM), URB597 (10 μM) 30 min prior to LPS: FAAH siRNA	PF and URB: PGE2/COX-2/iNOS/IL-6/IL-1β/MCP-1 ↓; IL-10/IL-4/Arg1/Ym1 no change; siRNA: PGE2/COX-2/iNOS/IL-6/IL-1β/MCP-1 ↓; IL-10/IL-4 ↑; Arg1/Ym1 ↑ w/o LPS	SR1/SR2/GW6471/GW9662/O1918 no effect	(81)
LPS (10 ng/mL) + IFNγ (10 U/mL) 3 h	N9	AM1241(10 μM) co-incubated	TNFα/iNOS ↓; Arg1/BDNF ↑	mitochondria/mtDNA/ATP/complex1 &4/Nrf1/Tfam/COX IV/MMP ↑; PGC-1α knockdown reversed	(82)
IFNγ (100 U/mL) + WIN55,212-2 (1 μM) 25 h	BV2	SR1 (1 μM) 1 h post IFNγ/WIN	IL-10 ↓; NO release ↑; MCP-1/TNFα/IL-1β/IL-6/IL-17/IFNγ/CX3CL1 ↑	IL-4/IL-10 ↓ and IFNγ/IL-17 ↑ in CD4+ T cells cultured in BV2 conditioned medium with SR1	(83)
	BV2	VCE004.8 (1 μM) 24 h	Arg1/PPARγ ↑	SR2 no effect; GW9662 no effect	(84)
LPS (25 ng/mL) 24 h	BV2	EEQ-EA (5-10 μM) or EDP-EA (5–10 μM) 4 h prior to LPS	IL-6/nitrite/cytotoxicity ↓; IL-10 ↑	AM630 (1 μM) reversed; eCB metabolites by CYP450 detected in brain; antiangiogenic	(85)
thrombin (20 U/mL) 48 h	rat primary MG	JWH133 (4 μM) 24 h post thrombin	IFNγ/CD86/CD68/IL-1β/TNFα ↓; TGFβ/IL-4/IL-10/CD206/Ym1 ↑	AM630 (1 μM) reversed; PKA inhibitor reversed; cAMP/P-PKA/Epac1 ↑	(86)
LPS (100 ng/mL) 24 h	rat primary MG	AEA (1 μM) 15 min prior to LPS	IL-6/COX-2/iNOS/NO ↓; IL-1β/IL-18/TNFα no change; IL-10/NGF ↑	AM630 but not AM251/CID1602 reversed NO release; AM630 reversed IL-18/TNFα/COX-2	(87)
LPS (50 ng/mL), IL-4 (10 ng/mL) + IL-13 (10 ng/mL), or TGFβ (20 ng/mL) 6 or 24 h	rat, mouse, or human primary MG	2-AG (1 nM) or AEA (1 nM) 24 h	2-AG but not AEA ↑ in M2a (IL-4/IL-13); AEA but not 2-AG ↑ in M2c (TGFβ); Arg1/SOCS3/CB1 ↑ by 2-AG; Arg1/SOCS3/CB2 ↑ by AEA	AM251 (1 μM) and AM630 (1 μM) 0.5 h prior to IL-4/IL-13 reversed Arg1; CB2 KO ↓ Arg1/phagocytosis	(88)
LPS (1 μg/mL) 12 or 24 h	C8B4, human primary MG	SMM-189 1 h post LPS	CD16/32 ↓; CD206 ↑; rod-shape ↑; round/amoeboid shape ↓; eotaxin/IP10/MCP-1/TARC/MIP-1β ↓	HU308/JWH133: CD16/32 and CD206 ↓; SR2: CD206 but not CD16/32 ↑	(89)
LPS (1 μg/mL) 24 h	BV2, mouse primary MG	JZL184 (1 μM) 1 h prior to LPS; MAGL overexpression	JZL: Fcγ-induced phagocytosis ↓; inflammatory cytokines/iNOS no change (primary MG); MAGL overexpression: Fcγ-induced phagocytosis ↑ (BV2)	phagocytosis Fcγ-mediated; MG132 reversed effects of MAGL; MAGL knockdown no effect	(90)
LPS (10 ng/mL) + IFNγ (10 U/mL) 24 h	N9 MG	AM1241 (5 μM) 1 h prior to LPS	Arg1/IL-10/BDNF/GDNF ↑; iNOS/IL-1β/IL-6/TNFα ↓	AM630 (10 μM) reversed; PKC inhibitor (10 μM) reversed	(91)
LPS (1 μg/mL or 0.1 μg/mL) 18 or 24 h	human primary or immortalized MG	SMM-189 (9.8 μM) 1 h post LPS or (13.4 μM) co-treated with LPS or IL-4	CD11b/CD45/CD80 ↓; IL-8/chemokines/IFNγ/IL-6/IL-12/IL-10 ↓; CD206 ↑ in IL-4 co-treated	LPS/IFNγ/IL-10/IL-4 ↑ CB2	(92)
LPS (50 ng/mL) + IFNγ (100 U/mL) 24 h	mouse primary MG and neuron mix	AEA (10 μM) co-treatment	IL-1β/IL-6 ↓; IL-10 ↑	ERK/JNK signal involved; CD200R ↑; neuron death ↓; CD200R KO and CD200 Ab reversed	(93)
TMEV infection at MOI (5 PFU/cell) 18 or 24 h	mouse primary MG	AEA (10 μM) co-treatment	IL-12/IL-23/IL-17A/NFκB ↓; IL-10 ↑	SR2 (1 μM) not SR1 (1 μM) reversed; Erk/Jnk inhibitor reversed; IL-10 Ab reversed IL-12/IL-23	(94)
LPS (50 ng/mL) + IFNγ (100 U/mL) 24 h	mouse primary MG	AEA (10 μM) co-treatment	NFκB/IL-12/IL-23/P-IκBα ↓; IL-10 ↑	ERK1/2/JNK/NFκB pathways involved; SR2 reversed; AEA treated conditioned medium down T-bet (Th1) but up GATA3 (Th2) in splenocyte	(95)

↓↑, *increased or decreased by eCB treatment, respectively; Ab, antibody; h, hour; KO, knockout mouse; MG, microglia.*

M2 Polarization Regulated by eCB

Regulation of M2 polarization by eCB and the sub-phenotype characterization of microglia induced by the eCB system have been extensively investigated by Dr. Guaza's laboratory since 2010 (95). When mouse primary microglia activated by LPS and IFNγ were co-incubated with AEA, expression of IL-10 was dose-dependently increased. This gene regulation was likely mediated by CB2, ERK1/2, JNK, and NF-κB but not by PI3K/Akt signaling pathways. In their subsequent report, neurotoxicity triggered by microglia was examined using a mixed culture of neurons and reactive microglia activated by LPS and IFNγ. Treatment with AEA reduced neuron toxicity, downregulated IL-1β and IL-6, and upregulated IL-10 and the CD200 receptor (CD200R), which is known to suppress the microglial inflammatory response and maintain the homeostatic state via interaction with the neuron-derived ligand, CD200 (96). Thus, CD200-CD200R axis enhancement by AEA may underlie its neuroprotective effects, and it may also shift microglial polarization toward the M2 phenotype and/or the homeostatic M0 state (93). In primary microglia activated by infection with Theiler's murine encephalomyelitis virus (TMEV), AEA treatment increased the expression of IL-10 and decreased the expression of the proinflammatory cytokines, IL-12p70 and IL-23. N-arachidonoylserotonin (AA-5HT), an endogenous cannabinoid that inhibits FAAH (97) and TRPV1 (98), also dose-dependently upregulated IL-10 in the TMEV model (94). Moreover, using rat primary microglia without activation, they found that both 2-AG and AEA at 1 nM concentrations were potent inducers of M2 markers, such as Arg1, which increased more than 20-fold (88). Of note, the higher concentration (100 nM) was counteractive to M2 marker induction. When the primary cultures were stimulated with the M2a-subtype inducers, IL-4 and IL-13, 2-AG levels but not AEA levels increased, and the expression of Arg1 and IGF1 increased as well. Administration of TGF-β shifted microglia toward an M2c subtype, indicated by an increase in SOCS3 expression and AEA levels but not 2-AG levels. The eCB metabolic enzymes are distinctly regulated among the two phenotypes: DAGLα was increased while MAGL was decreased in the M2a subtype; NAPE-PLD was increased while FAAH was decreased in the M2c subtype. Upregulation of the biosynthetic enzymes and downregulation of the degrading enzymes resulted in an increase in 2-AG and AEA levels in the M2a and M2c states, respectively. This study clearly demonstrated that the eCB system is tightly regulated by the M2 polarization sub-phenotype, and, in turn, M2 polarization is significantly regulated by endogenous eCB ligands. Consistently, another study showed that M2 polarization by the eCB system is critical under pathological conditions induced by LPS and/or IFNγ. Treatment with AEA in LPS-induced rat primary microglia downregulated IL-6, COX-2, and iNOS and reduced NO production, while IL-10 and NGF were increased dependent on CB2R (87).

Epoxyeicosatetraenoic acid-ethanolamide (EEQ-EA) and epoxydocosapentaenoic acid-ethanolamide (EDP-EA), which are the epoxide derivatives of eCBs catalyzed by CYP450, have recently been discovered in rat brain samples (85). Administration of these metabolites showed decreased IL-6 expression and nitrite production but increased IL-10 expression in LPS-activated BV2 cells; these effects were partially dependent on CB2R activation.

M2 Polarization Mediated by CB Receptor Agonists/Inverse Agonists

Microglia express both CB1R and CB2R; however, CB2R is more abundantly expressed in microglial cells (99), and its expression is further increased during activation *in vitro* and in disease animal models (23). Therefore, it is expected that CB2R plays a crucial role in the anti-inflammatory microglial response. Upregulation of the alternative M2 markers by CB2R activation in microglial cells has been reported (91). CB2R agonist AM1241 was shown to suppress the expression of proinflammatory cytokines, IL-1β, IL-6, and iNOS, in LPS/INFγ-activated N9 microglial cells. At the same time, there was an increase in the expression of Arg1, IL-10, and the neurotrophic factors BDNF and GDNF, which were significantly reduced by co-administration of the CB2R antagonist AM630 or the PKC inhibitor. In a recent follow-up study, AM1241 also showed an acceleration of mitochondrial biogenesis and an increase in the expression of mitochondrial transcription factors and genes, such as Nrf1, Tfam, and cytochrome *c* oxidase subunit IV, under the control of PPARγ coactivator-1α (PGC-1α) (82). This finding is quite intriguing given that the metabolic balance of energy in microglia/macrophages is drastically switched from the glycolytic pathway in the M1 state to the mitochondrial respiratory pathway in the M2 state (100). Mitochondrial biogenesis could be one aspect of M2 polarization regulation by eCB that could enhance mitochondrial oxidative phosphorylation. M2 phenotype modulation by eCB was observed in cells under other pathological conditions, including cells from an intracerebral hemorrhage model (86). Treatment with JWH133 upregulated the expression of several M2 markers, such as TGF-β, IL-4, IL-10, CD206, and Ym1, in microglial cells. M2 marker upregulation was reversed by the CB2R antagonist AM630. Other research groups tested the effect of a natural CB2 agonist, β-caryophyllene (BCP), which has been approved by the FDA as a food additive. IL-1β and TNFα were downregulated, and iNOS expression and ROS production were reduced in mouse primary microglia when 1 μM but not 5 μM of BCP was added 24 h prior to LPS treatment. In contrast, IL-10 and Arg1 were upregulated. As a result of increased Arg1 and decreased iNOS, generation of urea was increased, while that of NO was reduced. These changes clearly indicate that the metabolic balance of arginine was shifted toward the M2 phenotype (80). VE004.8 is a dual agonist for PPARγ and CB2R. Navarrete and colleagues investigated the pharmacological effects of this compound using several different cell types, including endothelial cells, RAW264.7 macrophages, and BV2 microglial cells, in a hypoxic environment (84). Results from the experiments using RAW264.7 and BV2 cells showed a consistent increase in Arg1 and PPARγ, and this increase was not affected by co-administration of the PPARγ antagonist, GW9662. Although CB1R expression is very low in microglia, its activation has also been reported to modulate microglial polarization (83). When BV2 cells

activated with IFNγ were co-incubated with SR141716A, a CB1R antagonist, the expression of TNFα, IL-1β, and IL-6 were upregulated. In addition, when IFNγ-activated BV2 cells were incubated with the CB1R/CB2R agonist WIN55212-2, co-incubation with SR141716A reduced the expression of IL-10 and increased the expression of inflammatory cytokines and Cx3cl1. Thus, CB1R-mediated modulation may also trigger a shift toward the alternative phenotype in microglia under certain culture conditions.

CB2R signaling blockade by inverse agonists does not always induce the inflammatory outcome; in fact, it has been shown to induce the anti-inflammatory response in some situations. A synthetic CB2R-selective inverse agonist, SMM-189 (101), was reported to downregulate the expression of several M1 markers, including CD16/32, IFNγ, IL-6, IL-8, and MCP-1, while upregulating M2 markers, such as CD206 and CD209 (89, 92). Of note, the microglia (C8B4 cells) that they used behaved in a contradictory manner compared to other reports; CD206 expression was upregulated by SR144528, a CB2R antagonist, but downregulated by the CB2R agonists JWH133 and HU308. In their earlier report, CD206 was increased by SMM-189 in the presence of IL-4 in human microglial cells, while IL-10 was decreased in primary human microglia (92). Thus, microglial M2 polarization by SMM-189 seems unclear in *in vitro* culture systems.

M2 Polarization Modulated by eCB Degradation Inhibitors

Inhibition of eCB-degrading enzymes can boost eCB signaling by increasing the levels of endogenous ligands that are available to suppress inflammation, but it is unclear how microglial polarization is altered. We recently reported on the anti-inflammatory effects of PF3845 and URB597, two commonly used FAAH inhibitors, and FAAH knockdown by siRNA in BV2 cells. Both pharmacological and genetic inhibition downregulated COX-2, iNOS, and proinflammatory cytokine expression; however, only siRNA knockdown of FAAH showed enhancement of M2 markers, such as IL-4 and IL-10, both in the presence and absence of LPS treatment (81). The discrepancy between the pharmacological inhibition and siRNA knockdown is unclear; however, different downstream pathways might be involved. Until now, there have been only a few studies examining the effects of eCB-degrading enzyme inhibitors on microglial alternative activation *in vitro*, and future studies in this area may help illuminate the reason for these inconsistencies.

EFFECTS OF ECB MODULATION ON MICROGLIAL POLARIZATION IN ANIMAL MODELS

Microglia are one of the central players in neuroinflammation linked to many neurological diseases (60, 102, 103). A substantial number of studies have examined whether modulation of microglial/macrophage activation was affected by the eCB system in preclinical animal studies. Herein, we review microglial polarization by eCB in animal models of neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), traumatic brain injury (TBI), and stroke related diseases. The results of these studies are briefly summarized in **Table 2**. Readers are encouraged to refer to several excellent review articles recently published regarding microglial activation and its potentially pathogenic role in AD (120), PD (121), and TBI (5, 122).

Microglial Polarization by eCB in MS-Related Animal Models

To our knowledge, the first reports of microglial M2 polarization by the eCB system in *in vivo* studies were published by Dr. Guaza's group (94) and Dr. Simeonidou's group (119). Using an experimental autoimmune encephalopathy (EAE) model created by PLP injection, Simeonidou's group studied the modulatory effects of 2-AG in the EAE model and showed that administration of 2-AG increased the number of ramified microglia, which resemble homeostatic microglia, and the number of $Arg1^+$ cells; however, the $iNOS^+$ cell population was unchanged (119). Consistent with *in vitro* culture studies (see above), administration of AEA reduced the expression of several proinflammatory cytokines, increased that of IL-10 in serum at 90 days post infection (dpi), and improved motor function in a demyelinating disease model induced by TMEV (94). In a recent study, the effects of 2-AG on microglial polarization were examined in the early phase of TMEV induction (7 dpi) (108). Proinflammatory cytokines, including IL-1β, TNFα, IFNγ, and iNOS, as well as chemokines and chemokine receptors, including Ccr2, Ccl2, Ccl3, and Ccl5, were substantially reduced by 2-AG administration. In contrast, the expression of Arg1 and IL-10 was increased several-fold. It was found that in addition to an increase in $Arg1^+/CD11b^+$ microglia/macrophages, the number of $Arg1^+/Iba1^-$ cells, which are putatively monocytic-myeloid derived suppressor cells (M-MDSCs) that have infiltrated the CNS, also increased. These results suggest that increased Arg1 expression was derived not only from microglia but also from infiltrated macrophages or M-MDSCs (108). In a recent study (104), 2-AG was subcutaneously injected into a TMEV animal model during the late stage of the infection (28 dpi) for 1 or 2 weeks. With this regimen, the pathological signature of demyelination [i.e., loss of $CC1^+$ cells, reduced myelin basic protein (MBP), and a high g-ratio] was significantly ameliorated after 60 days; however, modulation of the M2 phenotype was unclear: TNFα, IL-1β, and IL-10 were all upregulated after 35 days. Expression of both CD68 and CD206 were unchanged after both 35 and 42 days. The only gene associated with the M2 phenotype that was significantly altered after 42 days was BDNF, which was increased. This study implies that M2 phenotype polarization and pathological profile are not always correlated with each other (104). Although it is generally believed that CB2R plays a key role in the anti-inflammatory effects of the eCB system, CB1R overexpression in lumbar spinal cord delayed the onset of clinical symptoms and attenuated clinical score and demyelination in an MS model induced by MOG peptide immunization (114). Proinflammatory genes, including TLR4, IL-1β, IL-6, IL-17, and TNFα, and the key transcription factor

TABLE 2 | Effects of eCB modulation on microglial polarization in animal disease models.

Model construction/ animal	Disease	eCB treatment	M1/M2 phenotype	Other pathologies	Behavioral test	Antagonist test	References
Female SJL/J (4–6 wk), TMEV (2 × 10^6 PFU) right intracerebral injection	MS	2-AG (3.5 mg/kg) subcutaneous 28DPI daily	35DPI: Iba1 IR/TNFα/Il-1β/IL-10/Cxcl12 ↑; CD206/CD68 no change; MBP-loaded MG no change 42DPI: Cxcl12/BDNF ↑; MBP-loaded MG ↑; CD206/CD68 no change	35DPI: Sema3F/Napepld/Msr1 ↑, MBP/PLP/Olig2/CC1/OPC/Ccl2/CD47/Mgf-e8 no change; 45DPI: Sema3A/Sema3F/Napepld/Lamp1 ↑; CD47/Sirp1a/Mgf-e8 no change; Faah ↓; 60DPI: g-ratio ↓; $CC1^+$/OPC/MBP/PLP ↑; $Iba1^+$ ↓			(104)
Male C57BL/6 (3 mo), air blast (50 psi) left side	TBI	raloxifene (5 or 10 mg/kg) i.p. 2 h post TBI + daily for 14 days	$Iba1^+$ ↓; $CD16/32^+$ ↓; $CD206^+$ ↑ in right optic nerve; M1/M2 ratio ↓	Optic nerve axon count ↑; occulomotor nucleus loss ↓	visual activities improved		(105)
Male C57BL/6 (3 mo), air blast (50 psi) left side	TBI	SMM-189 (6 mg/kg) i.p. 2 h post TBI + daily for 14 days	$CD16/32^+$ ↓; $CD206^+$ ↑ in optic tract; $Iba1^+$ ↓ in retina	Axon bulbs ↓ in optic tract; optic nerve ↑; $GFAP^+$ ↓ in retina	visual function (contrast sensitivity) improved		(106)
Male APP/PS1 TG (8 mo)	AD	JWH015 (0.5 mg/kg) i.p. for 8wk daily	Iba1 IR/IL-6/TNFα/iNOS ↓; Ym1/2 ↑ in crtx but no change in hipp	Plaque # no change; dendritic spine complexity ↑ in cortex but no change in hipp	no change Morris water maze; improve novel object recognition		(107)
Female SJL/J (4-6wk), TMEV (2 × 10^6 PFU) right intracerebral injection	MS	2-AG (5mg/kg) or UCM-03025 (5 mg/kg) i.p. for 7 days	$Arg1^+$ $CD11b^+$ (MG/macrophage) ↑; activated MG morphology ↓; $Iba1^+$ ↓; Arg1/IL-10/IFNγ ↑; iNOS/TNFα/IL-1β/Ccr2/Ccl2/Ccl3/ICAM1/Csf1r ↓	$CD45^+$ infiltration ↓; $Arg1^+$ Iba1-(M-MDSC-like) ↑; $CD4^+$ T cell ↓; viperin/Bax/Casp3 ↓; Bcl2 no change	vertical motor activity improved	AM630 reversed more than AM251	(108)
Male S-D rat (300–400 g), vertebral artery occlusion and 10 min ischemia 24 h later	Four-vessel occlusion induced vascular dementia	Paeoniflorin (40 mg/kg) or HU308 (3 mg/kg) i.p. 15 min post occlusion then daily for 27 day	IL-1β/TNFα/IL-6/$CD68^+$/nitrite/iNOS/NF-κB/IκBα/mTOR ↓; IL-10/TGF-β1/Arg1/YM1/CB2/$CD206^+$ ↑	Neuronal damage in hipp CA1 ↓; P-IκBα/P-mTOR↓; P-PI3K/P-Akt ↑	spatial memory improved	AM630 reversed	(109)
Male strain unknown (8–10 wk), PMCAO (MCA cauterized) 24 or 48 h	stroke	JZL184 (4 mg/kg) i.p. immediately after PMCAO	TNFα/MMP9 ↓; IL-10 ↑	Infarct/edema ↓; improved neurological damage (bederson test)	sensorimotor function/muscle performance/ neurological deficit score improved	AM251 (3 mg/kg) did not reverse but improved some tests	(110)
Male C57BL/6 or CD-1 (12–16 wk), CCI 3 mm depression X 3 mm diameter convex tip	TBI	GP1a (3 mg/kg) or AM630 (5 mg/kg) i.p. 10 min post CCI	iNOS/TNFα/IL-6/IL-1β/Ccl2/Cxcl10 ↓; IL-10/Arg1 ↑; M1 type ($CD68^+$ $TNFα^+$ CD206-) ↓; M2 type ($CD68^+$ $IL10^+$ $CD206^+$) ↑ in macrophage/microglia ($CB2^+$/$CD11b^+$)	Edema ↓; cerebral perfusion ↑; CD45high macrophage ↓; CD45low MG no change	motor function/anxiety improved	AM630 no effects	(111)
Male S-D rat (250–300 g), 100 μl autologous arterial blood infusion into right basal ganglia	Intracerebellar hemorrhage (stroke)	JWH133 (1.5 mg/kg) i.p. 1 h post-surgery	Arg1/Ym1/CCL22/CD206/IL-4/IL-10/TGFβ ↑; CD32/CD86/CD68/IL-1β/iNOS/TNFα ↓	Apoptotic/damaged neuron ↓; P-CREB/P-PKA ↑; edema ↓	Neurological severity score/forelimb placing test improved	SR2 (3 mg/kg) i.p. 3 min prior JWH reversed; CREB KD reversed	(112)
Male S-D rat pup P7, 0.3 U bacterial collagenase infusion to right ganglionic eminence	GMH (stroke)	JWH133 treatment for 7 days	$Iba1^+$/BDNF/ramified MG/CX3CR1 ↑	$BrdU^+$ neuron/MAP2/nestin/NeuN/Tuj-1/NeuroD ↑; fiber bundle up in internal capsule		NeuN/NeuroD/Tuj-1/Dbcn/$Iba1^+$/$NeuroD^+$ ↓ by CX3CR1 knockdown	(113)

(Continued)

TABLE 2 | Continued

Model construction/ animal	Disease	eCB treatment	M1/M2 phenotype	Other pathologies	Behavioral test	Antagonist test	References
Male S-D rat pup (15–7 g), 0.3 U bacterial collagenase infusion to right ganglionic eminence	GMH (stroke)	JWH133 (1 mg/kg) 1 h post infusion i.p. 3–72h	$CD68^+$/CD68/CD86/iNOS/IFNγ/IL-1β/TNFα ↓; $CD206^+$/CD206/Arg1/Ym1/IL-4/IL-10/BDNF ↑			AM630 (1 mg/kg) reversed	(86)
Female C57BL/6 (8–10 wk), MOG35-55 (300 μg) subcutaneous injection	MS	CB1 Lentivirus (1.75×10^8 TFU) intrathecal injection in lumbar spinal cord 5 day prior to EAE	NFκB/Tlr4/IL-1β/IL-6/TNFα/IL-17A ↓; IL-10/NT3/BDNF/GDNF ↑; IFNγ no change in spinal cord at 28dpi	Demyelination/infiltration ↓; $IFN\gamma^+$/$IL\text{-}10^+$/$IL\text{-}17^+$ no change in $CD4^+$Tcells; $CD206^+$/$IL\text{-}10^+$ ↑ but $CD16/32^+$ ↓ in splenic $CD11b^+$ monocyte	Clinical score ↓		(114)
Male C57BL/6 (3 mo), MPTP hydrochloride (20 mg/kg) and probenecid (250 mg/kg) twice/week for 5 wks i.p.	PD	JZL184 (8 mg/kg) i.p. 5 d/wk for 5 wks	TGFβ/GDNF ↑ but no change IL-1β/IL-6/TNFα in Strtm; $Iba1^+$; ramified/small cell body sized MG ↑ in Strtm	2-AG/AEA ↑ in midbrain; TH^+ ↑ not $MAC1^+$ in SNpc; DAT/TH/TH^+/$GFAP^+$/$Iba1^+$ ↑ in strtm; cytoplasmic b-catenin ↓; nuclear b-catenin ↑ in Strtm	Improved motor function in MPTP model		(115)
Male C57BL/6 (8 wk), CCI 3 mm diameter X 1.5 mm depth left parietal crtx once	TBI	PF3845 (5 mg/kg) i.p. 30 min post CCI + daily	COX-2/$COX\text{-}2^+$/iNOS/$iNOS^+$ ↓ in crtx and hipp; $Arg1^+$ ↑ in crtx	AEA/2-AG/Synaptophysin/Bcl2/Hsp70/Hsp72 ↑; lesion volume/damaged neuron/APP ↓ in crtx; P-ERK/P-AKT ↑	Improved memory/fine motor skills/ anxiety	AM281 (3 mg/kg) and AM630 (3 mg/kg) reversed neuron damage/motor function/working memory	(116)
Male C57BL/6 (8 wk), CCI 3 mm diameter X 1.5 mm depth left parietal crtx once	TBI	WWL70 (10 mg/kg) i.p. 30 min post CCI + daily	iNOS/$iNOS^+$/COX-2/$COX\text{-}2^+$ ↓; $Arg1^+$ ↑	Lesion volume ↓; degenerated neurons ↓; BBB breakdown ↓; P-AKT/P-ERK/CB1/CB2 ↑	Motor function and working memory improved	AM281 (3 mg/kg) and AM630 (3 mg/kg) reversed neuron damage	(117)
Male Swiss (8–10 wk), permanent MCAO ligature of trunk before bifurcation for 15–24 h,	stroke	JWH133 (1.5 mg/kg) i.p. 10 min post MCAO	$Iba1^+$/IL-6/IL-12/MIP-1α/MCP-1/RANTES/iNOS ↓; IL-10/TGFβ/Ym1 ↓; COX-2/MPO/Arg1/IL-4 no change	Infarct ↓	Neurological severity score ↓ after 48 h	SR2 (3–5mg/kg) i.p. 3 min prior JWH reversed, CB2KO no effect of JWH133	(118)
Female SJL/J (4–6 wk), TMEV (10^6 PFU) right intracerebral injection	MS	AEA (3.5 mg/kg) infusion 83DPI for 7 days; AA-5HT (5 mg/kg) 78DPI for 12 d	IL-1β/IL-6/IL-12/IL-23/IL-17A ↓; IL-10 ↑ in serum/spinal cord	CD200/CD200R1 ↑	Improved motor function		(93)
Female SJL/6 (6–8 wk), PLP (150 μg) subcutaneous injection	MS	2-AG (100 μg) i.p. from D0 for 14 days	Ramified MG ↑; $Arg1^+$ ↑; $iNOS^+$ no change 22DPI	Axonal loss ↓; lymph node cells ↓; CB1/CB2 ↑			(119)

↓↑, increased or decreased by eCB treatment, respectively; crtx, cortex; DPI, days post injury; h, hour; hipp, hippocampus; i.p., intraperitoneal injection; IR, immunoreactivity; KD, knockdown; KO, knockout mouse; MG, microglia; MOI, multiplicity of infection; mo, month; P7, postnatal day7; PFU, plaque-forming unit; psi, pounds per square inch; S-D rat, Sparague-Dawley rat; SNpc, substantia nigra pars compacta; strtm, striatum; TG, transgenic mouse; wk, week.

NF-κB were downregulated specifically in the spinal cord but not in the brain and spleen. In contrast, there was an increase in IL-10^+ and $CD206^+$ microglial/macrophage cells and an increase in the neurotrophic factors NT3, BDNF, and GDNF in the spinal cord. These phenotypic changes indicate the potential for neuroprotective effects and axon repair. Recently, their follow-up study indicated that the use of SR141716A exacerbated EAE clinical scores and upregulated the expression of NF-κB and proinflammatory cytokines and chemokines. This finding was consistent with the notion that CB1R activation plays a key role in the eCB anti-inflammatory response in the MS model (83).

Microglial Polarization by eCB in Cerebral Hemorrhage and Stroke Models

Germinal matrix hemorrhage (GMH) is defined as damage to the brain resulting from the rupture of blood vessels within the subependymal germinal region of the ganglionic eminence in the immature brain. Neuroinflammation is deeply involved in the disease pathogenesis and progression. The therapeutic effects of the CB2 agonist JWH133 on microglial activation in the GMH animal model, which was created by intracerebral infusion of collagenase, have been extensively investigated. After a 24 h infusion, JWH133-administered animals showed attenuated edema and perihematomal tissue injury and improved motor and memory function (123). In addition, $Iba1^+$ and reactive microglia populations were reduced (124). In a subsequent report, they characterized time-dependent changes in M1 markers in perihemotomas and found that upregulation of M1 marker (IFNγ, IL-1β, TNFα, CD68, CD86, and iNOS) expression started relatively early (6–24 h post-injury) and was attenuated by JWH133. In contrast, regulation of the expression of M2 markers (IL-4, IL-10, BDNF, Arg1, Ym1, and CD206) was slightly delayed (24–72 h post-injury) and was enhanced by JWH133 (86). Thus, as shown in other brain injury models, microglial activation of the M1 phenotype was induced early on (6 h post-injury) and then downregulated, while that of the M2 phenotype was induced at a later time point with a potential peak at 24–72 h post-injury. This study demonstrated that the eCB system modulates both M1 and M2 marker expression in a time-dependent manner in the brain injury model. In a later study, they examined the effects of long-term treatment with JWH133 in the disease model and found that microglia adopted a ramified cell shape and showed increased expression of CX3CR1, the fractalkine receptor (113). CX3CR1 is not regarded as an M2 marker; however, upon binding to the neuron derived ligand, CX3CR1 suppresses microglial activation and enables microglia to return to the homeostatic state. These studies suggest that the CB2 agonist may shift the microglial phenotype to either the alternative (M2) or the homeostatic (M0) state depending on the experimental settings. The therapeutic effect of JWH133 was also investigated in another hemorrhage model created by the infusion of arterial blood into the basal ganglia (112). In this model, JWH133 was found to reduce brain edema, neurological scores, neurodegeneration, and apoptotic neuronal cells. M1 markers, including TNFα, IL-1β, CD68, and CD32, were all suppressed throughout the time period from 6 to 72 h post-injury, while M2 markers, including Ym1, Arg1, IL-4, IL-10, and TGF-β, were enhanced 24 h post-injury. Interestingly, since downregulation of phosphorylated CREB in the disease animal was reversed by JWH133, they created a knockdown animal model of CREB via intracerebroventricular infusion of siRNA and found that CD68 expression was upregulated and CD206 expression was downregulated in perihematomal tissue from the JWH133-treated animals. These results suggest that microglial classical (M1) and alternative activation (M2) marker expression may be regulated by CREB-mediated signal transduction (112).

Another study used a permanent cerebral ischemia model to examine the MAGL inhibitor, JZL184. Disease pathology, such as edema and infarct volume, was reduced by treatment with JZL184. In addition, TNFα and MMP9 expression was downregulated, while IL-10 expression was upregulated in the JZL184-treated group compared to the vehicle control. Interestingly, co-administration with CB1R antagonist AM251 did not significantly reverse the effects of JZL184 in some behavioral tests and pathologies (110). Paeoniflorin, an active ingredient in a traditional Chinese medicine, was reported to be a CB2R agonist that modulates the M2 phenotype evidenced by an increase in M2 markers, including Arg1, Ym1, IL-10, and TGF-β1, and a decrease in M1 markers, including IL-1β, IL-6, and TNFα, in a middle cerebral artery occlusion (MCAO) model. These modulatory effects were reversed by co-treatment with AM630 (109). However, it is unknown whether this compound is a CB2R agonist, despite its ability to increase CB2R expression. Another study examined the effects of JWH133 in the MCAO model (118). Intraperitoneal injection of JWH133 10 min prior to occlusion improved infarct volume and neurological severity score after 48 h. Several M1 markers tested (i.e., IL-1β, IL-6, iNOS, TNFα) and $Iba1^+$ cells in the ipsilateral region were suppressed between 15 and 24 h. In addition, M2 markers, including IL-10, Ym1, and TGF-β, were also downregulated, whereas Arg1 and IL-4 expression was not significantly changed. The authors hypothesized that CB2R activation shifts microglia toward the inactivated state and results in anti-inflammation (118).

Microglial Polarization by eCB in Neurodegenerative Disease Models

A plethora of studies have shown that microglial activation can be the cause of neurotoxicity and the development of neurodegenerative diseases such as AD, PD, and ALS. Therefore, several preclinical studies have examined potential treatments, including eCBs, to suppress microglial neuroinflammation in these disease models (125). However, only a few investigations have examined the role of eCB system modulation on microglial polarization. In a recent study using the APP/PS1 transgenic AD mouse model, administration of the CB2 agonist JWH015 for 8 weeks significantly decreased the expression of $Iba1^+$ cells and proinflammatory cytokines and increased the expression of YM1/2 in the cortex (107). However, anti-inflammatory effects were not observed in the hippocampus. In line with these microglial responses, performance in the novel object

recognition test associated with the cortex was improved, whereas performance in the Morris water maze test related to hippocampal spatial memory was not significantly improved. These results suggest that the CB2 agonist modulates microglial phenotype in a region-dependent manner. Aymerich's group studied the effects of JZL184, a MAGL inhibitor, on the MPTP-induced PD model (115). After intraperitoneal injection of JZL184 for 5 days a week over 5 weeks, dramatically decreased dopamine active transporter (DAT) and tyrosine hydroxylase (TH) expression in the PD animal was partially but significantly reversed. Moreover, TH+ neurons in the SNpc were increased. The number of Iba1$^+$ microglia with longer ramifications and a larger cell body increased. Neuroprotective striatal TGF-β and GDNF expression was increased, but inflammatory cytokines, such as IL-1β, IL-6, and TNFα, were not significantly changed. The observed upregulation of TGF-β and GDNF is not necessarily derived from microglia alone but also possibly from astrocytes since GFAP immunoreactivity in the region was significantly increased. Since β-catenin levels in the nucleus were increased, Wnt/catenin signaling may be also involved in the anti-inflammatory response by JZL184. Two behavioral tests, the pole test and the rotarod test, showed that motor function was improved by JZL184 treatment in the MPTP model but not in control animals (115).

Microglial Polarization by eCB in TBI Models

Two research papers regarding microglial polarization by eCB in a TBI model have been published by our laboratory. Our TBI model was created by controlled cortical impact (CCI), and two different inhibitors of eCB-degrading enzymes were tested. In the first paper, injection of WWL70, an inhibitor for ABHD6, which is one of the enzymes responsible for hydrolyzing 2-AG, was administered 30 min after the initial injury and then once a day until the end of session (117). The TBI animals showed memory deficits, motor dysfunction, a pathologically massive tissue lesion, and blood brain barrier breakdown. WWL70 not only attenuated these behavioral impairments and brain pathologies, but it also suppressed the expression of COX-2 and iNOS and dramatically increased Arg1 expression. These results indicate that the microglial phenotype was shifted to the M2 phenotype by WWL70. In the second paper, we examined PF3845, an inhibitor of FAAH, which is the main hydrolyzing enzyme of AEA in the CNS (116). TBI model animals were injected with the inhibitor in the same manner as described above. One and two weeks after surgery, working memory and motor coordination were improved by PF3845 treatment, and lesion volume and neurodegenerative neurons were reduced. These effects were likely mediated by both CB1R and CB2R. Moreover, COX-2$^+$ cells and iNOS$^+$ cells were reduced, and Arg1$^+$ cells were increased in the ipsilateral cortex by PF3845 treatment. The increase in Arg1 was found at 3 days and continued for at least 2 weeks post-injury. Thus, the two eCB-degrading enzyme inhibitors demonstrated therapeutic efficacy and the potential to modulate microglial phenotype. Our recent report shows that WWL70 inhibits not only ABHD6 but also prostaglandin E synthesis in BV2 microglia (126); these results suggest that eCB-independent mechanisms might also contribute to the therapeutic effect of WWL70 in the TBI mouse model. Another study examined the effects of SMM-189, a CB2R inverse agonist, on microglial phenotype in a TBI mouse model. Consistent with the *in vitro* study, CD16/32$^+$ cells were decreased while CD206$^+$ cells were increased by SMM-189 administration in the right optic tract 3 days after blast injury (106). Very recently, the same group showed that raloxifene, which is a CB2R inverse agonist (127) but also known as a selective estrogen receptor modulator (128), induced anti-inflammatory effects by modulating the M1/M2 microglial phenotype (105). However, whether microglial modulation is dependent on eCB has not been examined. In a recent study, CB2R agonist GP1a was examined in a TBI model induced by CCI (111). TBI-induced edema, anxiety, and motor dysfunction were ameliorated at 3 mg/kg of GP1a and to a lesser degree at 5 mg/kg. Moreover, CB2R activation by GP1a decreased Ccl2, Cxcl10, iNOS, TNFα, IL-6, and IL-1β and increased IL-10 and Arg1. The CD45^{low} microglia population was unchanged by either TBI or GP1a treatment, whereas CD45^{high} macrophage infiltration induced by TBI was reduced at 3 days post injury. When fluorescence-labeled macrophages were administered intravenously, CB2R immunoreactivity after TBI was correlated with increased fluorescence; this correlation suggests that the cells expressing CB2R in the CNS are mainly macrophages. Based on these observations, it was postulated that mainly the infiltrated macrophages are responsible for the increase in M2 marker expression by CB2 activation; however, the contribution of microglia cannot be dismissed (111).

PERSPECTIVES AND FUTURE ISSUES

A significant number of studies that examine microglial polarization by the eCB system have emerged in the last decade (**Table 1** for *in vitro* studies and **Table 2** for animal studies). Several studies showed that the therapeutic effects of the eCB system were mediated by CB1R, CB2R, non-canonical receptors GPR55/GPR18, and PPARs; however, CB2R activation is thought to play an indispensable role in eCB-mediated anti-inflammatory effects in several diseases models. Moreover, in terms of microglial phenotypic modulation, CB2R is the predominant regulator both *in vitro* and in animal models.

However, it is still unclear whether CB2R signaling, indeed, has the potential to commit microglia to the alternative (M2) phenotype *in vivo* because of technical limitations in the current approaches and the limited data available: commonly used methods, including gene or protein expression analyses or immunohistochemistry, provide a "snapshot" of the microglial activation state, but tracking changes at the individual cell level remains difficult. Therefore, whether classical activation (M1)-committed microglia can switch to the alternative activation (M2) phenotype or vice versa during disease development or drug intervention is still unknown. It may be possible to address this issue by monitoring live cells in animals using

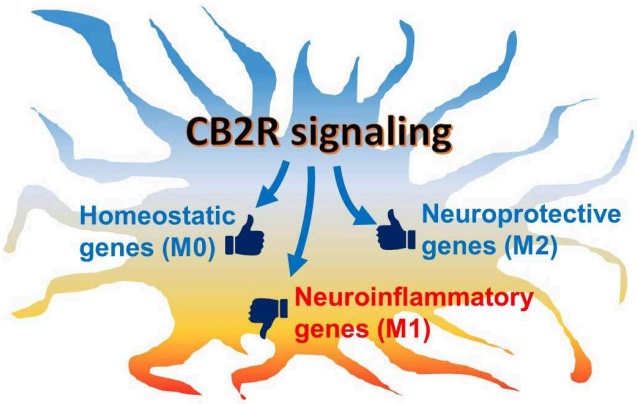

FIGURE 3 | Schematic hypothetical CB2R signaling effects on microglial phenotype. Microglia are activated under neuropathological conditions, in which neuroinflammatory genes are mainly induced while neuroprotective genes are also regulated. Under chronic conditions, the predominantly inflammatory activated microglia often cause neurotoxicity and neurodegeneration. When CB2R, which is abundantly expressed in microglia, is activated, its downstream signaling modulates the balance of gene regulation toward the neuroprotective function. In addition, homeostatic genes, which are associated with communication with neurons and synaptic activity, are also up-regulated to return the cell to physiological conditions.

two photon microscopy and genetically labeled or manipulated animals with fluorescent markers, such as Tmem119-EGFP transgenic mice (129–131). Second, the expression analysis of M2 gene markers is useful for alternative (M2) phenotype assessment. However, most of the studies described here have shown only a few markers positively upregulated. Therefore, it is uncertain if the upregulation of only a limited number M2 markers really indicates an acquired commitment to the alternative (M2) phenotype or if it indicates only a partial transition. More comprehensive investigation is needed in order to understand alternative (M2) phenotype modulation. In the last couple of years, several studies have used single-cell RNA-sequencing to investigate individual microglial gene regulation. These studies have consistently demonstrated the heterogeneity of microglial populations dependent on region, age, and pathological conditions (132). Although reactive microglial gene signatures that were evoked by immunostimulation and disease have been identified, none of them have matched the gene set of M2 markers (74–77). To fill the gaps in our understanding of microglial gene expression and subsets, we suggest further investigation, including pathohistological analysis with a stricter classification protocol using microglia-specific markers (i.e., $CD45^{low}$, Tmem119, and P2ry12) and multiple M2 markers rather than only one or two. Nevertheless, although the pure microglial phenotypes can be observed *in vitro*, the M1/M2 dichotomy is not pathophysiologically relevant since microglia in the brain would never receive only one cytokine but, rather, several environmental cues that modulate their phenotype in either direction. In fact, it was reported in some studies that both M1 and M2 gene markers are co-expressed in the same cells (73, 78). We hypothesize that individual microglia co-express neuroinflammatory (M1-type) genes and neuroprotective (M2-type) genes. Thus, eCB would not switch the microglial population from the M1-like phenotype to the M2-like phenotype; rather, eCB, together with microenvironmental cues, would shift the balance of expression between the two gene sets toward the neuroprotective function (**Figure 3**). In the future, further investigations, including transcriptomic studies, may reveal new gene markers for the neuroinflammatory M1 and the neuroprotective M2 gene sets.

In terms of morphological changes during microglial polarization, several studies using different animal models (113, 115, 119, 123) have shown that microglial morphology changes to a more ramified cell shape rather than a bipolar or amoeboid shape in disease models after eCB treatment. The latter morphology is thought to be related to classical (M1) activation though microglia have a ramified cell shape with a small soma when in the homeostatic (M0) state. In line with the morphological data, studies showed that eCB administration increased the expression of CD200R *in vitro* in a mixed neuron/microglia culture (93) and CX3CR1 in a stroke model (113), both of which are thought to be associated with alternative (M2) and homeostatic (M0) states (133). Although it remains uncertain whether the increase in homeostatic microglia is merely an epiphenomenon of terminated neuroinflammation, eCB signaling may directly shift microglial morphology toward not only the neuroprotective (M2) phenotype but also the homeostatic (M0) phenotype, in which microglia are known to have important physiological functions, which include synaptic pruning, synaptic plasticity modulation, and neuronal trophic support (46). The homeostatic state induced by eCB may play a role in neuron repair and restore synaptic activity, similar to the putative function of the neuroprotective (M2) phenotype. Further studies are necessary to elucidate the molecular mechanisms of microglial modulation by eCB and to define the classification of microglial phenotypes, including the homeostatic (M0) state, under pathophysiological conditions.

AUTHOR CONTRIBUTIONS

MT and SS contributed to literature search, writing and editing the manuscript, and preparing tables and figures. YZ contributed to writing and editing the manuscript.

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Playing Mahjong for 12 Weeks Improved Executive Function in Elderly People With Mild Cognitive Impairment: A Study of Implications for TBI-Induced Cognitive Deficits

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Background: Mild cognitive impairment (MCI) is common among elderly people. So far, effective treatment that can stabilize or reverse the cognitive decline associated with MCI is lacking. Recent studies suggest that playing mahjong may improve attention and memory in elderly people. However, its effect on executive function remains unknown.

Methods: 56 elderly people (74.3 ± 4.3 years of age) with MCI from the First Social Welfare the First Nursing Home of Nanchong were randomized into mahjong and control groups ($N = 28$, each group). Subjects in the mahjong group played mahjong three times a week for 12 weeks, while people in the control group assumed normal daily activity. Executive function was evaluated using the Montreal Cognitive Assessment—Beijing (MoCA-B), the Shape Trail Test (STT), and the Functional Activities Questionnaire (FAQ) before the study and then at 6 and 12 weeks after mahjong administration.

Results: There were no baseline differences in MoCA-B, STT, and FAQ scoring between the two groups. The MoCA-B, STT, and FAQ scores, however, improved significantly in the mahjong group but not in the control group after the 12-week mahjong administration. Significant correlations were also found between STT and FAQ scores.

Conclusions: Playing Mahjong for 12 weeks improved the executive function of elderly people with MCI. Because Mahjong is a simple, low-cost entertainment activity, it could be widely applied to slow down or reverse the progression of cognitive decline in people with MCI, including those with traumatic brain injury.

Keywords: mahjong, elderly, executive function, mild cognitive impairment, activities of daily living (ADL), TBI

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INTRODUCTION

Mild cognitive impairment (MCI) is a critical transient period of cognitive decline between normal aging and early dementia. Between 10 and 20 percent of adults above the age of 65 are diagnosed with MCI, and approximately 10 percent of MCI adults progress to Alzheimer's disease annually (1). The decline in executive function in elderly people with MCI is particularly obvious. The activity and executive function of the prefrontal cortex is significantly reduced with aging (2). Executive function is the advanced cognitive function for completing tasks and/or overcoming difficulties

involving prefrontal cortex-mediated working memory and reflection, planning, organization, and management (2). The decline in executive function may have a negative effect on the instrumental activities of daily living (IADL). Marshall et al. as well as others, have shown a significant correlation between executive function and the ability to complete IADL in elderly with MCI (3). Mansbach et al. reported that most elderly with MCI were deficient in their abilities to perform IADL (4). Executive function was a stronger predictor for forecasting IADL dependence than memory, particularly for the performance of complex finances, the performance of complex cooking, and the ability to remember events (4). Executive function was more closely related to instrumental and advanced ADL than to basic ADL (5). Another study has shown that intervention to stabilize/improve executive function in the early stages of dementia may delay the decline of IADL and improve the quality of life of elderly people (6). Thus, there is a need to explore effective interventions that can improve executive function in elderly people.

Recent studies show that intellectual activities and hobbies involving mental workload can have therapeutic effects on cognitive function in elderly people (7). Cheng et al. reported progressive improvement in cognitive performance after patients who were affected by an early stage of dementia played mahjong (8). Playing mahjong has also been found effective in improving short-term memory, attention, and logical thinking in both middle-aged and elderly people (9). Improved episodic memory after playing mahjong may involve the activation of selective and divided attention, inhibition of interfering stimuli, and mobilization of manipulation skills (10). An enriched environmental, emotional stimulation, and interpersonal interactions during mahjong activity may also play a role in the reactivation of neural circuits in an aged brain.

Mahjong is a popular form of social entertainment for elderly people in China. It has win-or-lose gambling-like characteristics and is played among four players. In order to win, the participants need to focus and coordinate visual, mental, and manual activities. These repeated activities may improve executive function in aging.

Playing mahjong requires four players sitting around a square table with raised edges. A mahjong set includes 136–152 tiles (depending on the version of the game) and three dice. Players mix the tiles (called washing) and place the tiles face-down, building them into blocks two layers high. They take turns throwing the dice and receiving tiles according to the number thrown, as well as sorting and arranging their own tiles into some desired spatial sequence. They then take turns drawing one tile from the face-down pile and discarding a piece. It is important to memorize the tiles played and to predict other players' moves and use this information to build a strategy to maximize one's chances of winning. In completing sets, combinations of three tiles can be formed as either three of a kind (a pong) (one variation is four of a kind) or a sequence of three numbers in a row of one suit (a chee). During play, every player has to pay attention to certain tiles when they become available to form a pong (win the game). The player may call "pong" any time, even when it is not his/her turn if he/she has two of a kind and the latest discard gives him/her a pong. The player calling "pong" takes the discard and reveals all three pieces (11) (https://corp.mahjongclub.com/basic-rules; https://www.thesprucecrafts.com/how-to-play-mahjong-411827; https://www.youtube.com/watch?v=7WygnpfFbMQ; https://www.youtube.com/watch?v=tRCb_LOkEmQ).

So far, however, no study has directly examined the effects of mahjong play on executive function in elderly people with MCI. In this study, we examined executive function and IADL in older adults with MCI who played mahjong for 12 weeks.

METHODS

Subjects and the Nursing Home

People 65 years of age or older were recruited from the First Social Welfare Nursing Home of Nanchong City, Sichuan Province, China. The nursing home was selected for this study because (1) the lifestyle of elderly people in the nursing home was relatively simple and homogeneous; 2) the nursing home always had enough participants to play mahjong games at a given time.

Inclusion Criteria Included

- 65 years of age or older;
- Diagnosis and confirmation of mild cognitive impairment, including reports of cognitive decline by self or nurses; assessment with the Beijing version of the Montreal Cognitive Assessment (**MoCA-B**), (cut-off range: 17–23 [illiterate], 20–24 [elementary school], 20–25 [middle- to high school and above]); Clinical Dementia Rating (CDR), 0.5–1.0;
- Prior experience and knowledge of how to play mahjong, but had not played mahjong in the past 6 months;
- Free of a handicap and/or disability that could interfere with mahjong playing.

Exclusion Criteria Included

- Inability to participate in mahjong due to disability (e.g., visual impairment, hearing impairment, other severe diseases);
- A psychiatric history;
- A neurologic disorder that could affect cognitive function;
- Inability to comply with the time frame of this study;
- Suffering from major depression diagnosed with Geriatric Depression Scale-15 (GDS-15).

People with depression were excluded because depression is an independent negative variable affecting IADL ability and neuropsychological functioning including executive control and episodic memory in people with MCI (12). Depressive symptoms are associated with cognitive decline and aging (13).

GDS-15 has a set of 15 depression-related questions for evaluating depression in elderly people. A Chinese version of this scale has good reliability and validity in assessing elderly people (14, 15). In this study, GDS-15 scores of 8 or above indicate a

Abbreviations: ADL, activities of daily living; FAQ, functional activities questionnaire; GDS-15, geriatric depression scale 15-item version; IADL, instrumental activities of daily living; MCI, mild cognitive impairment; MoCA-B, montreal cognitive assessment (Beijing edition); STT, shape trail test; TMT, trail making test.

possible depression status, and people who scored 8 or above were therefore excluded from the study.

The Clinical Dementia Rating (CDR) scale was applied to measure the grade of MCI and to exclude with CDR-2 or CDR-3 in elderly (16).

The sample size calculation was performed based on data (effect size is 0.7, αis 0.05, power is 0.8)from literature using the statistical program G*Power3.1. The sample of each group (mahjong or control) comprised at least 34 subjects, with a total of 68 subjects needed to achieve statistical representativeness. 69 participants who fulfilled the eligibility criteria were randomized into a mahjong group (35) and control group (34), with a total of 56 subjects completing the whole experiment.

All study procedures were conducted in accordance with the Helsinki Declaration of 1975 and were approved by the Institutional Review Board of Nanchong Central Hospital. Written informed consent forms were obtained from all participants prior to the start of the study, and no monetary reward was offered to participants of the study.

EXPERIMENTAL PROCEDURE

The participants were randomized into a mahjong group or control group according to a computer-generated random number table with a 1:1 allocation ration. All participants were assessed over the course of three visits. During the first visit, details of the study were presented and written informed consent was obtained. Patient information sheets were also obtained and neuropsychological tests were performed. The final two assessment visits included neuropsychological tests, conducted during the sixth week and the twelfth week of the experiment.

INTERVENTION

The mahjong group was instructed to play mahjong three times a week for 1 h each time for 12 weeks based on previous reports (17, 18). Each mahjong game was played by a group of four players randomly assembled at the time. The project researchers were responsible for scheduling and managing the mahjong event. If one or more of the scheduled players could not show up for a game for any reason, another resident would serve as the temporary substitute. The control group includes those residents who did not participate in any form of mahjong activity during the trial period except their normal daily activities. Participants were instructed to record their daily activities through a daily life sheet following the staff's instruction given to them 1 week before the study. Participants in the mahjong group played mahjong Monday, Wednesday, and Friday afternoons at two o'clock in the same location to increase their compliance. To control bias, all participants were asked to keep a record of regular daily activity on the daily life sheet under the supervision of the staff.

MEASURES

The values of the Montreal Cognitive Assessment Scale—Beijing (**MoCA-B**) (19), the Shape Trail Test (STT) (20) and the Functional Activity Questionnaire (FAQ) (21) were assessed before and at 6 and 12 weeks after the start of the study by researchers who were not involved in the management of mahjong activity.

THE MONTREAL COGNITIVE ASSESSMENT—BEIJING (MoCA-B)

MoCA, first developed by Nasreddine, a clinical research center for neurology at Charles LeMoyne Hospital in Canada, was used to screen elderly people with mild cognitive impairment (22). The scale includes eight cognitive domains with a best potential score of 30 points. Each correct answer accounts for 1 point, whereas a wrong answer or no answer accounts for 0 points. **MoCA-B** was translated and revised by Wen in 2008 to include some cultural modifications (23). **MoCA-B** is one of the most widely used MoCA versions in China with high sensitivity (83.8%) and specificity (82.5%).

THE SHAPE TRAIL TEST (STT)

The STT is a series of Arabic numerals, combined with Chinese culture (20, 24) and consists of two parts. Part A consists of 25 digits. The subject is asked to connect the numbers as pre-instructed. Part B also contains 25 digits, but each digit appears twice, in both a circle and a square, and need to be alternately connected by the subjects as pre-instructed. That test also shows the acceptable level of area under the curve (AUC = 0.835), the sensitivity (84.6%), and specificity (66.7%) of STT in elderly people (> 65 years) with an education level <12 years.

THE FUNCTIONAL ACTIVITIES QUESTIONNAIRE (FAQ)

The Functional Activities Questionnaire (FAQ), as a self-reported questionnaire, is used to differentiate the independence of instrumental activities of daily living (IADL). The FAQ is composed of ten items including simple finances, complex finances, shopping, games, simple cooking, complex cooking, current events, tracking media, remembering events, and transportation; the ability to perform each activity is rated from 0 (normal) to 3 (dependent) with a score range of 0–30. The higher the score, the more serious the damage of IADL function.

Statistical Analysis

Data were analyzed using SPSS 22.0 software. The baseline differences and the differences after 6 and 12 weeks of mahjong intervention in **MoCA-B**, STT, and FAQ scoring between the two groups were analyzed using independent *t*-test and/or repeated measures analysis of variance as appropriate. A paired *t*-test was used to analyze the before-and-after treatment differences within each treatment group. $P < 0.05$ was considered statistically significant.

RESULTS

A total of 69 elderly people were recruited to participate in this study. The subjects were randomly assigned to the mahjong intervention group ($n = 35$) or to the control group ($n = 34$). During the intervention period, seven individuals from the mahjong group and six from the control group were excluded, leaving 28 in the mahjong group and 28 in the control group. The process for the selection of subjects is described in **Figure 1**.

The average age of all participants was 74.3 ± 4.3; the male-to-female ratio was 15:41 (26.8 to 73.2%), the education time was 5.7 ± 3.9 years, and the proportion of illiteracy (level of or under junior high school, and senior high school) was 21.4, 64.2, and 14.3%, respectively. The values in our present study are consistent with the recent report of elderly Chinese patients in another study by Jiang, 2008. The mahjong group did not differ significantly from the control group in age (74.4 ± 3.9 vs. 74.2 ± 4.8 years) ($p > 0.05$), years of education (5.6 ± 3.7 vs. 5.9 ± 4.2) ($p > 0.05$), proportion of female (78.6, vs. 67.9%) ($P > 0.05$), or CDR (0.88 ± 0.59 vs. 0.84 ± 0.62) ($p > 0.05$) (**Table 1**).

A trend level difference existed in the baseline Montreal Cognitive Assessment (**MoCA-B**) between the mahjong (21.11 ± 2.22) and control (22.18 ± 2.39) groups (t/P-values =-1.74/0.09). Trend level differences were also found after 6 weeks (21.3 ± 1.9

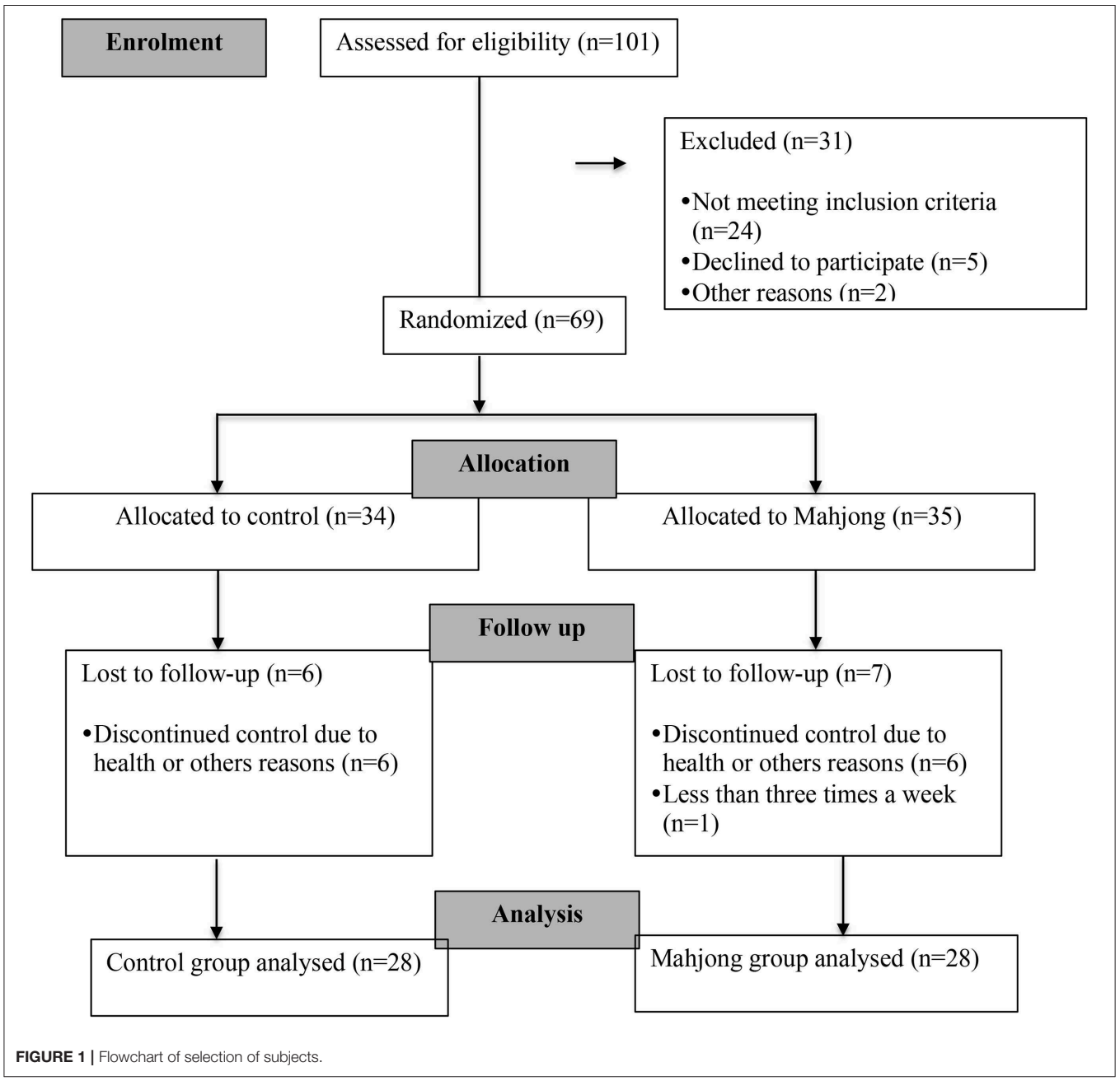

FIGURE 1 | Flowchart of selection of subjects.

vs. 22.1 ± 2.3, *t*/*P*-values =-1.4/0.15) and 12 weeks (22.8 ± 1.7 vs. 22.0 ± 1.9, *t*/*P*-values =1.63/0.11) of mahjong playing. However, an opposite direction of change in **MoCA-B** occurred between the two groups, i.e., a small but steady increase in the mahjong group and a small but steady decrease in the control group after 6 and 12 weeks of mahjong playing. The control group analysis showed that the MoCA-B score was significantly improved in the mahjong group after 12 weeks of mahjong intervention (22.8 ± 1.7) compared to the baseline (21.11 ± 2.22) ($P < 0.01$) and 6-week values (21.3 ± 1.9) ($P < 0.01$). But no such change was found in the control group (**Table 2**).

Similarly, no significant difference was found in STT between the mahjong and control groups at the baseline (573.1 ± 113.8 vs. 559.3 ± 95.9, $P > 0.05$), 6 weeks after mahjong intervention (555.1 ± 115.0 vs. 561.4 ± 102.0, $P > 0.05$), and 12 weeks after mahjong intervention (535.7 ± 111.7 vs. 565.5 ± 93.0, $P > 0.05$). Group time analysis showed continuously reduced STT scores in the mahjong group after 6 weeks (555.1 ± 115.0) ($P < 0.01$) and 12 weeks (535.7 ± 111.7) ($P < 0.01$) of mahjong playing compared to the baseline STT scores (573.1 ± 113.8). But no such reduction in STT scores was found in the control group (**Table 2**).

Compared to the baseline FAQ values (17.89 ± 4.64), the FAQ score decreased significantly and continuously in the mahjong group after 6 weeks (16.9 ± 4.5) ($P < 0.01$) and 12 weeks (15.6 ± 4.8) ($P < 0.01$) of mahjong intervention but not in the control group. The FAQ scores of the mahjong group became significantly lower in the mahjong group than in the control group after 6 weeks (16.9 ± 4.5 vs. 19.5 ± 3.3, $P < 0.05$) and 12 weeks (15.6 ± 4.8 vs. 19.9 ± 3.6, $P < 0.01$) of mahjong playing (**Figure 2**).

The relationships between the scores of the MoCA-B and STT and the scores of the FAQ are shown in **Table 3**. Significant correlations were found between MoCA-B and FAQ and between FAQ and STT.

TABLE 1 | Demographics of the participants ($N = 56$).

	Mahjong	Control	Total	*P*
Gender, (women %)	78.6%	67.9%	73.2%	0.375
Age, year (mean ± s.d)	74.4 ± 3.9	74.2 ± 4.8	74.3 ± 4.3	0.879
Education, year (mean ± s.d)	5.6 ± 3.7	5.9 ± 4.2	5.7 ± 3.9	0.814
GDS (mean ± s.d)	3 ± 1.56	2.86 ± 1.7	2.93 ± 1.64	0.748
CDR (mean ± s.d)	0.88 ± 0.59	0.84 ± 0.62	0.86 ± 0.6	0.826

GDS-15, Geriatric Depression Scale; CDR, The Clinical Dementia Rating.

DISCUSSION

In this study, we evaluated the effects of mahjong playing on executive function in elderly people with MCI by using three scales, the MoCA-B, STT, and FAQ.

MoCA-B is a reliable screening tool for detecting different types of MCI, including amnestic MCI and non-amnestic MCI (25). According to a recent study of elderly Chinese participants with MCI, the cut-off values of MoCA-B for illiterate people (<1 year of education) with MCI is 17–23 points, for people with elementary school education, the boundary values are 20–24 points, and for people with junior high school education and above, the boundary values are 20–25 points (26).

The Trail Making Test (TMT) is one of the most sensitive and popular scales for testing executive function in people with MCI (27). In the test, subjects need to shift the focus of attention between various external stimuli when facing two cognitive tasks. The ability to shift tasks is a main component of executive function. Because TMT is based on the Latin alphabet, this limits its application in Chinese-speaking populations. STT is based on the Trail Making Test (TMT), which was developed for people who speak Chinese as a first language (20).

The Functional Activities Questionnaire (FAQ), as a self-reported questionnaire, is used to evaluate the independence

TABLE 2 | Changes in **MoCA-B**, STT-B, and FAQ scores (mean ± s.d) after 6 and 12 weeks of mahjong intervention.

	Baseline	6 weeks after intervention	12 weeks after intervention	t/*P*-value base vs. 6 w	t/*P*-value base vs. 12 w	t/*P*-value 6 w vs. 12w
MoCA-B						
Mahjong	21.11 ± 2.22	21.3 ± 1.9	22.8 ± 1.7	−1.000/0.326	−6.971/0.001**	−6.162/0.001**
Control	22.18 ± 2.39	22.1 ± 2.3	22.0 ± 1.9	0.493/0.626	0.895/0.379	0.593/0.558
t/*P*-value	−1.74/ 0.09	−1.47/0.15	1.63/0.11			
STT-B						
Mahjong	573.1 ± 113.8	555.1 ± 115.0	535.7 ± 111.7	8.88/<0.001**	8.59/0.001**	5.79/ 0.001**
Control	559.3 ± 95.9	561.4 ± 102.0	565.5 ± 93.0	−0.28/0.783	−1.71/0.66	−0.45/0.098
t/*P*-value	0.49/ 0.62	−0.22/0.83	−1.084/0.283			
FAQ						
Mahjong	17.89 ± 4.64	16.9 ± 4.5	15.6 ± 4.8	5.50/<0.001**	5.44/<0.001**	2.86/<0.01**
Control	19.36 ± 3.81	19.5 ± 3.3	19.9 ± 3.6	−0.87/0.39	−1.89/0.07	−1.8/0.083
t/*P*-value	−1.291/ 0.20	−2.61/0.012*	−3.74/0.00**			

**P < 0.05, difference between mahjong and control groups or between different times after mahjong intervention within each group; **P < 0.001, difference between mahjong and control groups or between different times after mahjong intervention within each group; base, baseline; STT-B, the Shape Trail Test-B; FAQ, Functional Activities Questionnaire; MoCA-B, Montreal Cognitive Assessment—Beijing.*

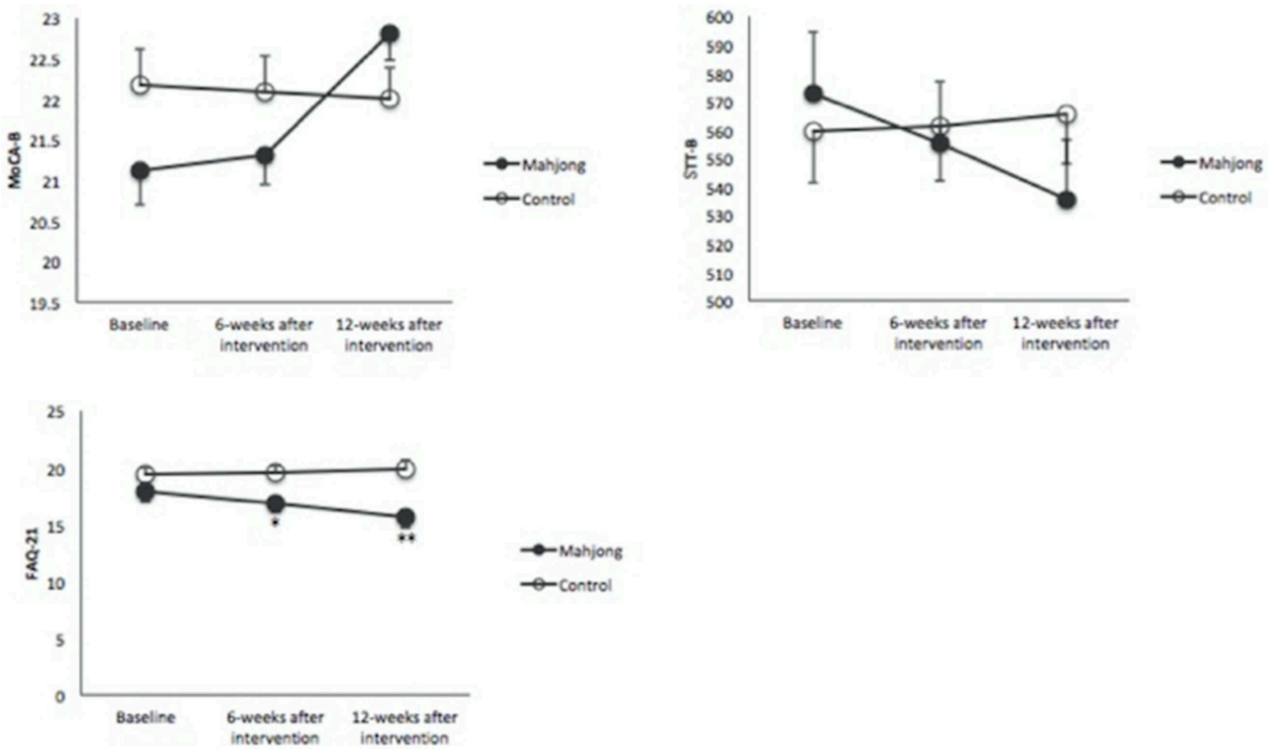

FIGURE 2 | Changes in the Montreal Cognitive Assessment–Beijing (MoCA-B), the Shape Trail Test-B (STT-B), and Functional Activities Questionnaire-21 (FAQ21) among the studied groups. A significant effect of time is observed on the FAQ21 between the two groups after 6 and 12 weeks, while no significant changes were observed in MoCA-B and STT-B. *$p < 0.05$, **$p < 0.001$ significant between groups at point of assessment.

TABLE 3 | Correlations between FAQ, MoCA-B, and SST-B before and after 6 and 12 weeks of mahjong playing of all participants ($N = 56$).

	FAQ: baseline	FAQ: 6 weeks	FAQ: 12 weeks	MoCA-B: baseline	MoCA-B: 6 weeks	MoCA-B: 12 weeks	STT-B baseline	STT-B 6-week	STT-B 12-week
FAQ: baseline	1								
FAQ: 6 weeks	0.956**	1							
FAQ: 12 weeks	0.872**	0.923**	1						
MoCA-B: baseline	−0.583**	−0.480**	−0.444**	1					
MoCA-B: 6 weeks	−0.563**	−0.465**	−0.456**	0.931**	1				
MoCA-B: 12 weeks	−0.495**	−0.490**	−0.569**	0.766**	0.759**	1			
STT-B: baseline	0.681**	0.603**	0.566**	−0.494**	−0.483**	−0.362**	1		
STT-B: 6 weeks	0.703**	0.653**	0.615**	−0.471**	−0.444**	−0.364**	0.956**	1	
STT-B: 12 weeks	0.720**	0.676**	0.671**	−0.436**	−0.424**	−0.388**	0.957**	0.937**	1

***Correlation is significant at the 0.01 level (two tailed).*

STT-B, the Shape Trail Test-B; FAQ, Functional Activities Questionnaire; MoCA-B, Montreal Cognitive Assessment—Beijingß.

of instrumental activities of daily living (IADL). Nitrini (28). reported that a FAQ score ≥6 indicates a functional impairment. FAQ is more sensitive in differentiating subjects with MCI from those without MCI when compared with other self-reported IADL scales (29).

Executive function consists of inhibition—responding appropriately to the needs of a task and/or specific objectives with controlled behaviors, thoughts, emotions and attention, and updates that allow for the retaining and manipulating of information to external tasks or stimuli—and cognitive flexibility, which allows one to modify one's behavioral response to external stimuli (30). Executive function plays an important role in completing complex activities like housekeeping, laundry, meal preparation, medication management, shopping, and transportation; the simultaneous activation of frontal cortex circuitry is involved in executive function (31).

It was first reported in 2006 that playing mahjong can significantly improve the cognitive function of patients with dementia (18). In a separate study, these authors also showed that mahjong playing had produced better outcomes than tai chi

exercise in improving the cognition function of elderly people. Lu et al. reported that playing mahjong can improve short-term memory, attention, and logical thinking in elderly people (9). However, these studies have not examined the effects of mahjong on executive function and ability of life activity.

Our current study focused on the executive function and activity of daily living and showed that playing mahjong improved the executive function of elderly people with MCI. This finding may reflect the fact that playing mahjong is mentally and intellectually challenging. In order to win the game, participants need to concentrate as well as judge and predict the next moves of others in order to select the best strategies to win. Eye and hand movements are also required to play mahjong. All these activities may have mobilized cognitive reserves in the brain and thus enhanced executive function.

Recent studies suggest reserved neural plasticity in the structure and function of the prefrontal lobes of elderly people. Training elderly people to perform a series of cognitive tasks involving working memory and integrated cognition delayed the shrinkage of the prefrontal cortex and improved white matter integrity, functional connection, and the differentiation of neural networks (32–35).

The significantly improved cognitive function after 12 weeks of mahjong playing in elderly people with MCI is consistent with previous reports that playing mahjong has significant effects on memory, attention, and ability to think (36). Our results, however, differ from a 2014 report by Cheng et al. that shows no difference between the mahjong group and the control group after mahjong intervention (8).

This difference may reflect the fact that we used MoCA-B in our study, whereas Cheng et al. used MMSE in their study. It is known that MoCA-B is more sensitive than the MMSE in evaluating the cognitive function of elderly people in the Chinese population (37). MoCA-B covers a wider range of cognitive deficits, whereas MMSE is suitable for detecting memory and language impairment. The application of MMSE in evaluating executive and visual–spatial impairment is also limited. It has been reported that MoCA-B has a higher sensitivity (78%) than MMSE (67%) in detecting early MCI (22).

In this study, we observed subjects with MCI whereas Cheng's team studied subjects with dementia who have fewer cognitive reserves and are more resistant to treatment than those with MCI (38). Thus, subjects with MCI may have more cognitive reserves to effectively engage in mahjong activity and activate the cerebral cortex than subjects with dementia.

We also studied the relationship between executive function and instrumental daily living ability. The significant correlations between severe executive dysfunction and a worsening ability of daily living in this study are consistent with previous reports (3, 39). Activities of daily life include the critical cognitive ability of executive function tasks including complex problem solving, attention shifting between tasks, inhibiting irrelevant information, recalling lists of items, and sustaining attention on tasks (40).

Increasing evidence supports a correlation between MCI and a decline in everyday functioning, which includes basic and instrumental ADL, self-care tasks, and living independently in a community setting (5, 40). Correlations between apathy, depression, memory and executive functioning, dependence in IADL, and falling and hospital readmissions have also been reported, (12, 40).

Because executive function is projected in the prefrontal cortex, which is sensitive to aging-related brain atrophy and traumatic brain injury (TBI), it would be interesting to know if the present findings can be applied to prevent or reverse the decline in prefrontal cortex functional activity and the ability of daily living of TBI patients (41). It is tempting to speculate that playing mahjong could stimulate activity and restore some of the lost functions of prefrontal cortex and thus improve the executive function and instrumental activities of daily living in TBI patients because improved social interactions are important for restoring executive function and instrumental activities of daily living in subjects with TBI (42). When participating in multi-person games like mahjong, the participants are involved in social interactions and therefore executive functions may be synchronized or boosted among the participants. These social interactions may be critical for strengthening the neural network and improving physical and executive functions (43, 44). Findings suggest that the playing of mahjong can likely prevent loneliness and be beneficial for psychological well-being (45). Playing Mahjong can improve hand–eye coordination and manual dexterity, as evidenced by quickened speed of finger activity and shortened response time. A Japanese study confirmed that the flexibility of hand function affects executive function in elderly people and that improved physical function by means of playing mahjong is also reflected in the enhanced activity of the hand (46).

Although it is not clear yet whether the benefits of playing mahjong are due to regular human interaction with peers or due to playing mahjong itself, the extensive engagement of human interaction could be the most critical factor in producing the beneficial effect. It is known that loneliness and social isolation are major determinants of mental well-being, especially among older adults (47). One recent review suggests that narrative activity-based social interventions (as opposed to dancing, gardening, or other physical activities) can bring about positive well-being on social and health-related measures in older people living in nursing homes or similar institutions (48).

However, mahjong is a four-player gambling game, which originated in China and is played in Chinese communities across the world, so financial problems are particularly concerning for older adults or TBI patients, since they are likely to be on fixed incomes or not working (49). In addition, prolonged mahjong activity could increase the risk for serious health conditions like hypertension and heart disease because of exposure to both direct and secondhand smoke. Playing mahjong also often involves money, which can be a stressful activity, especially when financial losses, marital discord, or other social problems may increase the risk for chronic diseases like diabetes and hypertension (50). There are case reports that mahjong could cause reflex seizures that are possibly induced by non-verbal cognitive tasks (51).

This pilot study has limitations. The number of participants is small because of drop-out, and no long-term follow-ups were conducted. Although the self-reported FAQ was used

to evaluate ADL, is the questionnaire is considered not as accurate as performance-based evaluations because it lacks sound psychometrical properties and its potential over- or underestimation of functional ability (4, 5). Future research could be done to determine the mechanisms of mahjong on executive function in elderly people with MCI and to ascertain the effects of long-term mahjong game on executive function in TBI patients.

CONCLUSION AND IMPLICATIONS FOR TBI

This study showed that playing mahjong for 12 weeks could improve executive function and the ability of daily activity in elderly people with MCI. Considering that many people with TBI also suffer cognitive deficits and executive dysfunction, the present findings suggest that mahjong as a cognitive game may be a potential method to improve these cognitive deficits and the activities of daily living in people with TBI. The time of participation and health conditions need to be considered when the mahjong playing method is practiced in TBI patients because of possible disadvantages.

DATA AVAILABILITY STATEMENT

The raw data is not publicly available. However, the raw data could be available by the corresponding author upon reasonable request.

ETHICS STATEMENT

All study procedures were conducted in accordance with the Helsinki Declaration of 1975 and were approved by the Medical Ethics Committee of Nanchong Central Hospital. Informed consent was obtained from all participants prior to enrollment into the study.

AUTHOR CONTRIBUTIONS

BZ, HZ, YP, and GX conceived the study. HZ, YP, CL, HL, and ZC contributed to the experimental implementation, participant enrollment, evaluation, follow-up, and data collection. HZ and GX contributed to the data analysis, literature search, and manuscript preparation. All authors have read and approved the manuscript.

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Brain Injury and Dementia in Pakistan: Current Perspectives

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Alzheimer's disease (AD) is the most common form of dementia, accounting for 50–75% of all cases, with a greater proportion of individuals affected at older age range. A single moderate or severe traumatic brain injury (TBI) is associated with accelerated aging and increased risk for dementia. The fastest growth in the elderly population is taking place in China, Pakistan, and their south Asian neighbors. Current clinical assessments are based on data collected from Caucasian populations from wealthy backgrounds giving rise to a "diversity" crisis in brain research. Pakistan is a lower-middle income country (LMIC) with an estimated one million people living with dementia. Pakistan also has an amalgamation of risk factors that lead to brain injuries such as lack of road legislations, terrorism, political instability, and domestic and sexual violence. Here, we provide an initial and current assessment of the incidence and management of dementia and TBI in Pakistan. Our review demonstrates the lack of resources in terms of speciality trained clinician staff, medical equipment, research capabilities, educational endeavors, and general awareness in the fields of dementia and TBI. Pakistan also lacks state-of-the-art assessment of dementia and its risk factors, such as neuroimaging of brain injury and aging. We provide recommendations for improvement in this arena that include the recent creation of Pakistan Brain Injury Consortium (PBIC). This consortium will enhance international collaborative efforts leading to capacity building for innovative research, clinician and research training and developing databases to bring Pakistan into the international platform for dementia and TBI research.

Keywords: TBI, dementia, Alzheimer's disease, Pakistan, aging, road traffic accidents, violence

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia, accounting for 50–75% of all cases, with a greater proportion of individuals affected at older age range. A single moderate or severe traumatic brain injury (TBI) is associated with accelerated aging and increased risk for dementia (1–3). The 2014 Alzheimer's Association Facts and figures Guidelines include TBI as a risk factor for AD along with advanced age, sex, family history of AD, positive Apo-e4 allele, cardiovascular disease, social, and cognitive engagement and education (4). Currently, 58% of the world's population aging with dementia live in low-middle income countries, but by 2050 this will rise to 68%. The fastest growth in the elderly population is taking place in China, Pakistan, and their south Asian and western Pacific neighbors (5). However, current clinical assessments are based on data collected from Caucasian populations from relatively wealthy backgrounds (3, 6–9), giving rise to a "diversity" crisis in brain research. This lack of ethnic diversity means that: (1) there is a lack of data that could teach us how AD progresses in populations with distinct backgrounds, especially in terms of political conflict, violent crime (including domestic and gender-based violence) and environmental hazards, and (2) there is little understanding about the predictors of brain or mental health that can be generalized from Caucasians to other ethnic groups. In this paper, we provide an overview of TBI and dementia incidence, management, and services available in a low-middle income country such as Pakistan. We also specifically point to a dire need of neuroscientific research in both TBI and AD in Pakistan. We conclude by presenting our recent initiative, Pakistan Brain Injury Consortium (PBIC), which seeks to improve the research and clinical services in Pakistan by bringing in international expertise, promoting research into TBI causes and treatment, training of staff, and creating awareness around TBI importance and care.

TRAUMATIC BRAIN INJURY AND DEMENTIA

A TBI occurs when the head is injured by a blow or penetration of an object resulting in brain damage. These injuries can be due to falls, road traffic accidents, athletic activities, firearms accidents, or assaults. Based on severity, TBI can be categorized as mild, moderate, or severe. Mild TBI would mostly result in concussions that are temporary and not life threatening while severe TBI may result in long periods of unconsciousness, coma, or even death. Although reports of mild TBI patients returning to baseline (pre-injury) functioning 1 year post-injury have been documented, 7–33% of these patients experience persistent symptoms (10). TBI occurs frequently in young people and is the most common cause of disability and death between the ages 1 and 45 (11). Annually, 10 million people are affected by TBI (11) and based on American Association of Neurological Surgeons about 1.7 million cases of TBI occur only in the US every year and 5.3 million people live with disabilities caused by TBI in the US, alone (12). The majority of those with TBI will recover in a matter of weeks (13). However, 10–30% will experience a persistent set of symptoms lasting for months, even years (14–16). Cognitive, sensory, and affective complaints erode the quality of life for these patients (17, 18), and such sequelae has been collectively labeled as persistent post-concussion syndrome (19–21).

A single blow to the head classified as a moderate or severe TBI is associated with progressive cognitive decline leading to dementia [reviewed in (1, 2)]. The recent attention given to Chronic Traumatic Encephalopathy (CTE; formerly known as dementia pugilistica) encountered in military personnel, veterans and in those who participate in contact sports, has raised much public concern. These links are particularly disturbing because they are associated with many alarming features. The rate of recovery from "mild" TBI is likely to be different for older adults and perhaps also impacting their quality of life, which may be much different in younger patients. Co-morbid conditions such as post-traumatic stress disorder (PTSD) and depression may prolong the chronic symptoms of TBI resulting in cognitive decline and dementia (22). Populations with TBI in areas with sociopolitical conflict (such as blast victims during suicide bombing and political riots), in addition to military personnel and veterans, might be at greater risk for accelerated cognitive decline and dementia as they grow older.

Jordan (23) provides an extensive review of the spectrum of chronic traumatic brain injury in sports, of which the most clinically pertinent are CTE, chronic post-concussion syndrome, and chronic neurocognitive impairment (NCI). Briefly, CTE represents the long-term neurologic consequences of repetitive mild TBI and is secondary to progressive tauopathy (24, 25). There are several risk factors associated with the development of CTE but exposure to contact sports is currently the most validated one. Dementia-like cognitive difficulties are commonly observed as the disease progresses and the pathophysiology of CTE is also well-defined. Chronic post-concussion syndrome is clinically distinct from CTE and has an acute onset related to the single TBI event. However, the most pertinent chronic TBI sequelae to the development of age-related dementia is what Jordan (23) classifies as chronic NCI. Although vague, it encompasses a large variety of symptoms that are a result of sports-related (perhaps blast-related as well) repetitive TBI. The symptoms may manifest within a year or even years after the event. It has no established relationship with CTE and can be described by neuropsychological testing (26). Most studies compared the performance in patients with NCI with either healthy controls or with performance prior to the event to ascertain decrements in performance. Specifically, impaired episodic memory has been reported in Jockeys after injury (27), and boxers with APOE ε4 allele have more neurological impairment than those without (28). Perhaps most direct evidence comes from McAllister et al. (29), who reported deficits in new learning, verbal learning, and memory in collegiate contact sport athletes at post- compared to pre-season. Cognitive deficits such as naming and word finding in visual/verbal episodic tasks are also documented in aging retired National Football League (NFL) players, and such deficits were correlated with white matter abnormalities on MRI (30). Volumetric MRI abnormalities, particularly reduced volume in caudate,

hippocampus, and amygdala, have been reported in boxers and mixed martial artists (31). Interestingly, Singh et al. (32) reported reduced hippocampal volumes in football players compared to healthy controls that were inversely correlated with football exposure. Reduced glucose metabolism was also observed in positron emission tomography (PET) scans in posterior cingulate cortex, parieto occipital lobes, frontal lobes, and cerebellum in retired boxers (33). Despite growing neuroimaging evidence, current TBI guidelines in the US do not recommend imaging as a diagnostic tool at the acute stage of mild and moderate TBI. Nevertheless, most diagnostic and treatment studies of TBI and dementia done in developed countries regularly utilize neuroimaging techniques.

Such a trend, albeit important to understanding the neurobiology of TBI and its association with cognitive decline, places developing countries at a disadvantage due to the lack of cutting-edge neuroimaging and clinical resources. Indeed, the prevalence of TBI and resulting psychiatric complications and disabilities constitute a huge burden on the economy and resources in developing countries like Pakistan. Hence, the goal of this paper is to present the current status of TBI and dementia incidence and the management of these health problems in Pakistan.

INCIDENCE OF BRAIN INJURY AND MANAGEMENT IN PAKISTAN

Razzak et al. (34) conducted a systematic review of brain injury incidence and risks in Pakistan from the perspective of a low-middle income country (34). In this review, they pointed out that The Eastern Mediterranean region of the World Health Organization, which includes Pakistan, has some of the highest death rates from injuries such as Road Traffic Incidents (**Figure 1**) and political conflicts [146,000 deaths and 2.8 million injuries just from road traffic crashes (36, 37)] (**Table 1**). **Figures 2**, **3** depict the Road Traffic Mortality Rates (RTM) across South Asian countries, with Pakistan reporting highest RTM at 25.5 per 100,000 population, even higher than India (44). Injuries caused 42 deaths per 100,000 population or 6% of all deaths (45). Specifically, injuries contribute about 11% of all deaths above the age of 60 years; 57% of all injuries occur among 15–59 year olds, with males (8 vs. 4% females) being more likely to suffer injuries (46, 47). Risk factors also include the lack of organized prehospital and hospital based trauma care (48). Additionally, domestic violence against women is a significant problem in Pakistan; the rates of physical and sexual violence estimated to be as high as 80 and 77%, respectively (42). In terms of political conflict, Pakistan is among the world's top five countries most affected by terrorism (49). The country had over 12,000 terrorist attacks between 2009 and 2016, resulting in 16,526 deaths. Suicide terrorist events, where civilians are victims in almost all events, make up 74.1% of deaths and 93.8% of those injured (49). Terrorist attacks and violent clashes among different political parties during the 2013 general elections led to 298 deaths and 885 injuries between January 1 and May 15 of that year alone (48). The province of Sindh—primarily Karachi was the most affected by both terrorist attacks and incidents of political violence (34). Between January and April, 2018, Lady Reading hospital Peshawar admitted an average of 281 patients per month—a city prone to Taliban violence (43). Although acute care is readily available via excellent ambulance networks (34, 39), there are no follow-up rehabilitation services available. Additionally, there is no current patient registry for follow-up, biobank or any infrastructure available to provide care and education to the aging population, compounding the long-term burden of TBI.

Similar to the US, children and youth are the age groups most affected by the TBI in Pakistan also (40, 41, 50, 51). The major cause of TBI in youth is road traffic accidents (40, 41); while in children, it is falls from a certain height (50, 51). Another rapidly increasing cause of TBI in Pakistan is the penetrating brain injuries (PBIs) occurring due to gunshots or other firearms (38, 52–54). The increase in such injuries is attributed mainly with the increase in level of violence and terrorism due to bomb blasts and suicidal bomb attacks (54) (Note: We expect this rate of PBI occurrence to have slowed following much improved security condition since 2016, however, no recent formal study has been published to provide such statistics), These injuries are broadly divided into three categories (52); (1) Tangential with gunshots glancing off the skull without entering, (2) Penetrating with gunshots entering the skull, and (3) Perforating with gunshot entering and exiting the skull. Penetrating and perforating injury patients are at a higher risk of mortality compared to tangential injury patients (51). PBI patients need surgeries more than other TBI patients and lack of neurosurgical facilities and neurosurgeons increase the mortality rate of PBI (52) patients. Management of PBI differs a lot from the non-penetrating brain injury and "Guidelines for Management of Penetrating Brain Injury" is very useful to handle such injuries (53). Sports related head injuries, especially because of the most popular sport of Cricket in Pakistan, are also common and can be fatal (55). However, not many sports related cases are reported simply because of the lack of awareness about them in the society (56).

In addition to the damage caused by TBI to the brain immediately or within a few days after the injury, there are chances of developing neurological issues and psychiatric disorders (11). Pituitary dysfunction is also very common among TBI survivors with moderate and severe TBI, which may have neurological consequences and can result in increased morbidity (57). Modifications in molecular mechanism can also occur in TBI patients increasing the chances of epilepsy and Alzheimer's disease (58).

TBI cases are increasing rapidly in Pakistan with the increase in population, poor safety considerations, and frequent incidents of terrorism. However, available infrastructure for diagnosis, treatment provision, and follow-up care is not adequate. There are many reasons for this lack of facilities but the major reasons are lack of resources such as trained medical staff and state-of-the-art medical equipment. Based on a published report there were only 35 neurosurgical centers and only one neurosurgeon per 1.37 million people in Pakistan (52). Furthermore, even these centers do not have enough ambulances and trained paramedics to transport the TBI patients from the site of injury

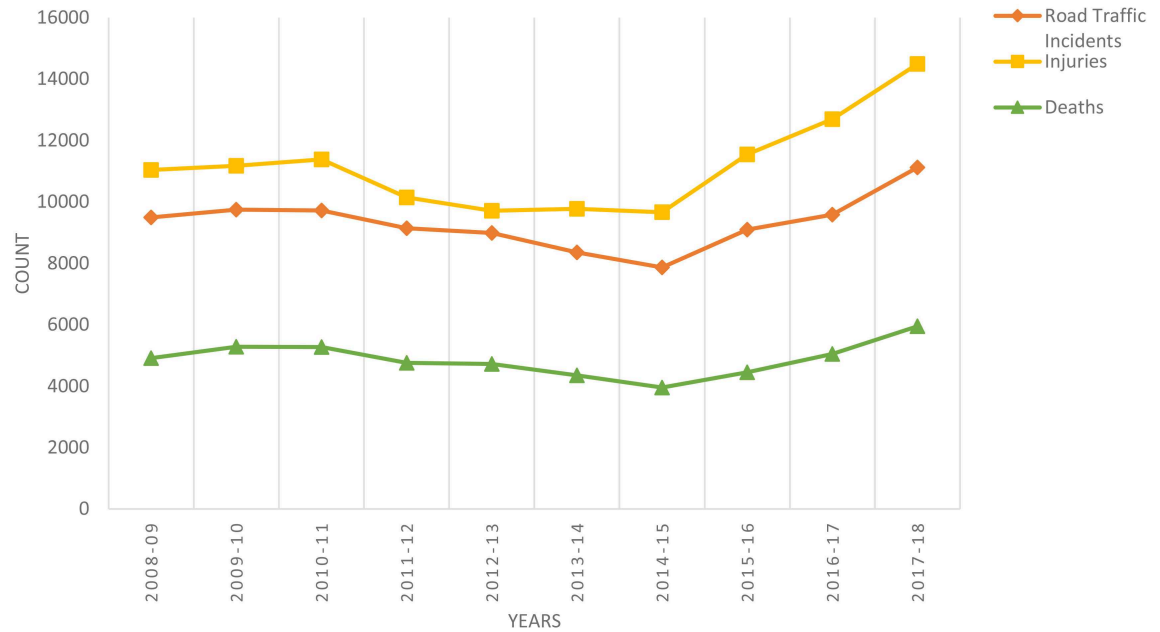

FIGURE 1 | Number of injuries and deaths due to Road Traffic Incidents (RTI) in Pakistan (35). Date represents information taken from: http://www.pbs.gov.pk/sites/default/files/tables/Traffic%20Accidents%20%28YEARLY%29.pdf.

TABLE 1 | Brain injuries and deaths due to TBI in Pakistan.

Year/Duration of data collection	Data source	Brain injuries	Deaths	Mechanism(s)	Reference(s)
1990–1993	Neurosurgical units in Karachi and Quetta	100	52	Missile Injuries (gunshot and ballistic missiles)	(38)
1993–1996	Edhi ambulance transportation, Karachi	4,091	2,400	Violence	(39)
1995–1999	Neurotrauma Centers in different areas all over the country	260,000	46,800	Road traffic accidents, Fall from Height, Assault, Agriculture Injuries, Sports Injuries, Fall from Train	(40)
2007–2011	Combined Military Hospital, Quetta	1,056	83	Road traffic accidents, falls, gunshot wounds, social violence, bomb blast, sports related, mine blast, splinter injury	(41)
2013	Global Terrorism Index, 2016	885	298	Terrorist attacks and violent clashes	(42)
2018 (Jan–Apr)	Lady Reading Hospital, Peshawar	1,125	–	Multiple	(43)

to these centers and to collect all the necessary information regarding any such injury. A multicenter TBI emergency care study reported out-of-pocket costs might be one of the major causes of TBI-based deaths or disabilities (59). Almost two-third of the population earned $2 a day per head in 2015 so affording the conveyance to the hospital (∼$8) and subsequent CT scan (∼$16) remained out of reach for many (59). In addition to the lack of resources, there is a lack of awareness about the importance of TBI patients' care immediately after the injury. Pakistani society also has unusual perceptions and sensitivities regarding the safety measures. For example, in Saeed et al. (60), the authors report that the female pillions, involved in TBI incidents, admitted not wearing helmets while riding a motorcycle and said that they would do so if they were male. Their reasons for not doing so was that they would look odd and societal pressure due to it being uncommon for females.

DEMENTIA AND ALZHEIMER'S INCIDENCE AND CURRENT MANAGEMENT IN PAKISTAN

Pakistan is the sixth most populated country in the world and currently has an estimated 150,000–200,000 patients with dementia (61). Life expectancy has increased in Pakistan in general leading to an increase in prevalence of dementia from

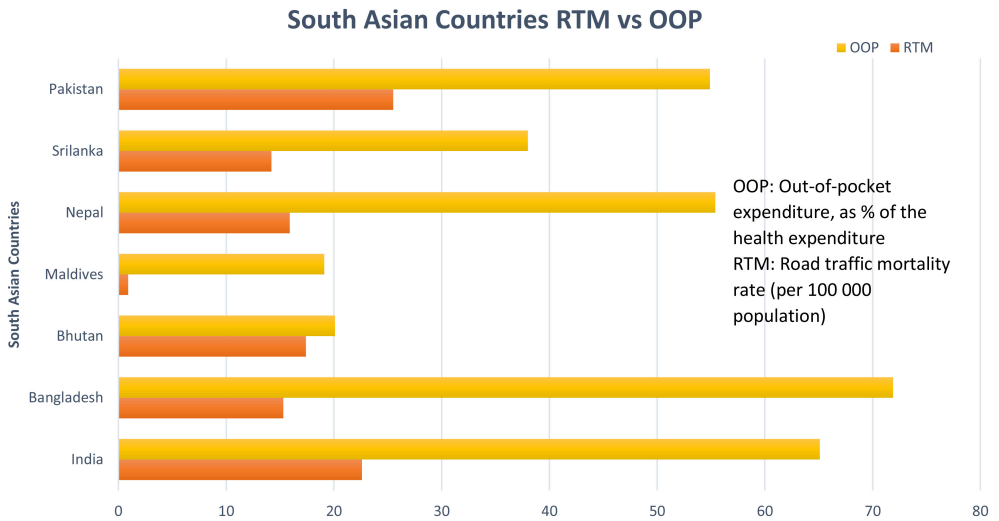

FIGURE 2 | Road Traffic Mortalities (RTM) rates and Out-of-pocket Expenditure across South Asia. OOP, Out-Of-Pocket (OOP) expenditure, as % of the health expenditure; RTM, Road Traffic Mortality rate (per 100,000 population). Data reported from Pakistan is from 2012 and for the rest of the countries is from 2016 [Data Source: World Health Organization (WHO)] (44).

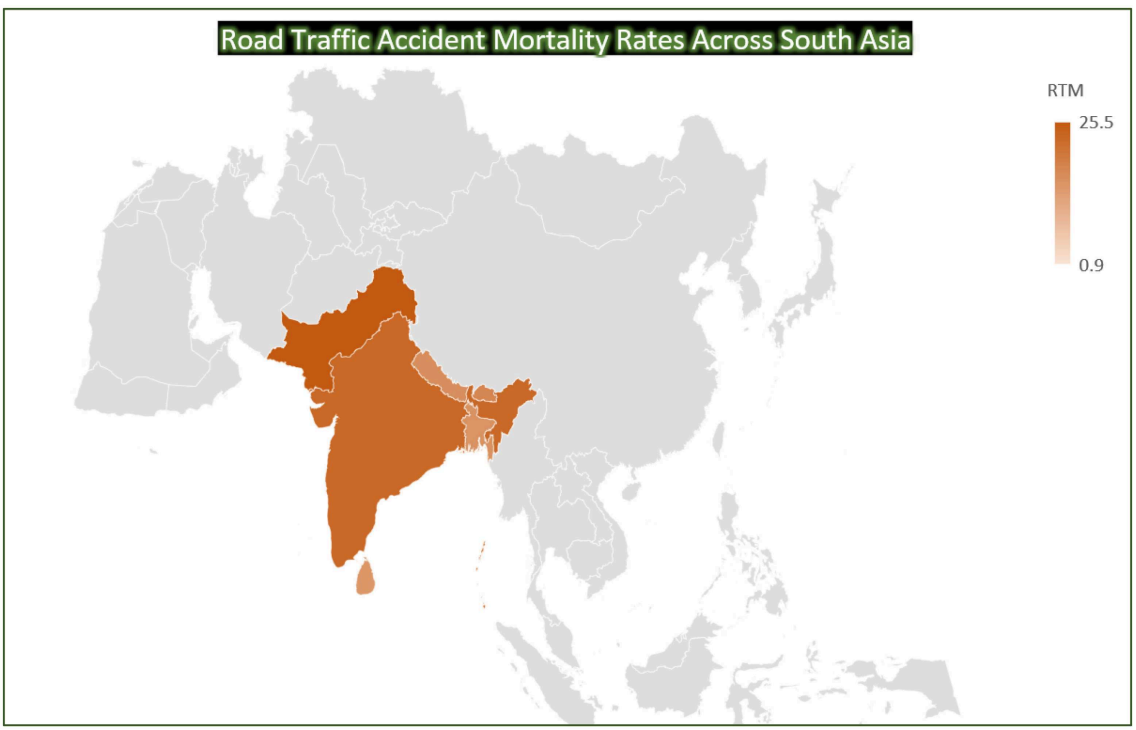

FIGURE 3 | Road Traffic Accident Mortality Rates Across South Asia. Road Traffic Mortality rate (RTM) per 100,000 population (44) (Map Template Courtesy: Bing).

2 to 6% in persons older than 65 years of age (62). Treatment focuses on behavioral and caregiver issues and management of this older population poses an economic challenge. Alzheimer's disease International is leading a dementia registry with Shifa International hospital in Islamabad, and a roll-out of national dementia guidelines. In the absence of a geriatric medicine subspecialty in Pakistan, dementia care falls under neurology and psychiatry's domain. Khan et al. (52) provides alarming numbers: one dementia trained specialist, one dementia registry, one dementia research center, two academic research clinics, and one dementia day care center in the entire country of Pakistan—a country of 197 million people (63). Of the 1,086 AD clinical trials conducted across the world, none are being conducted in Pakistan (63). The solution to this paucity of clinical research

and care infrastructure must include international collaborative efforts, training of clinicians, and researchers in sub-specialties of neurology and psychiatry, large longitudinal studies including clinical trials with genetic, biomarker and neuroimaging measures, and an effort to translate and validate psychological instruments (52, 61). Higher Education Commission (HEC), a national body that oversees all aspects of tertiary education and research in Pakistan, recognizes these needs and recommends deepening research collaboration across the globe and reforming research funding frameworks (64).

DISCUSSION

Our review demonstrates the lack of resources in terms of speciality trained clinician staff, medical equipment, research capabilities, educational endeavors, and general awareness in both the fields of dementia and TBI. In terms of clinical follow-up and existing registries, Pakistan National Emergency Departments Surveillance Study [Pak-NEDS (2010–2011); (59)] and Road Traffic Injury Research and Prevention Center [RTIRPC (2007–2017); (65)], are two databases developed previously. However, both databases are no longer collecting data but are available for recruitment. There are currently no biobanks available for brain injury or dementia in Pakistan either. Despite the state-of-the-art clinical research facilities available at the top ranking hospitals, such as AKU, these biobanks need to be developed, with database protocols and an emphasis on clinical and research staff training and community outreach.

In order to mitigate the road safety immediate concerns, the Sindh Governor Road Safety Advisory Board recommended the formation of National Road Safety Council (NRSC) to establish the National Road Safety Action Plan (66). The key implementation points were to establish a Road Traffic Injury Research and Prevention Center (RTIR&PC), a Road Crash Investigation system (involving traffic police, urban road network, insurance companies, and trauma registries), road safety, discipline, and compliance system, speed management, emergency services, and rehabilitation, improving motorcycling safety, implementing better licensing and helmet standards, and installing better road safety conditions for motorcyclists. However, this action plan was created in 2008 and there has been no updates in its implementation to date.

In order to improve the current situation of TBI treatment and care in Pakistan there is also a need to first create awareness about its importance in the society (56, 60). Strict measures should be taken to ensure the implementation of the traffic laws for e.g., to wear helmets. Number of neurosurgical centers, ambulances, and trained paramedics needs to increase (59). In addition to increase in neurosurgical centers, home-based caregivers must be trained to take care of TBI patients (67). To avoid long-term neurological and psychiatric disorders, regular checkups of the TBI patients after the injury can be done (57). This is where follow-up databases can really help.

Although we are limited by reporting no research data in the present review we hope our findings can be used to conduct a larger study in future. Our team has determined that Pakistan has an amalgamation of unique risk factors that lead to brain injuries such as lack of road legislations, terrorism (including suicide bombing), political instability, and domestic and sexual violence (34). Recent evidence further clarifies the molecular mechanisms underlying TBI that trigger amyloid precursor protein (APP) and Tau cleavage mediating AD pathology in animals (68). Additionally, the relationship of TBI and AD has shown to be quite complex and the presence of TBI leads to misdiagnoses of AD, interferes with treatment plans and makes research studies difficult to interpret (69). However, Pakistan lacks state-of-the-art diagnostic assessment of dementia and its risk factors, including neuroimaging of brain injury and aging that are extremely limited in Pakistan due to the inadequate infrastructure and limited training of clinicians (63). To help collaborative efforts, publications, and increasing awareness, we have established PBIC. The primary goal of PBIC will be to enhance collaborative efforts internationally and nationally through education, research, and publications on existing datasets. Specifically, we will teach emerging scientists to acquire and analyze data with high quality control and precision, and promote capacity building through training and educational endeavors. We believe that the large population in Pakistan provides a unique opportunity to yield high throughput research studies. Although low in numbers, a network of high-quality hospitals that are open to collaborate in developing local expertise in research and clinical care. In addition, Pakistan is one of the most philanthropic countries with substantial number of high net-worth individuals who can be invited to provide resources. Our ultimate goal is to bring Pakistan in the international arena for clinical neuroscience research and education and provide the data collected to global brain consortiums.

AUTHOR CONTRIBUTIONS

MA, MP, and AR contributed as primary authors including the concept, collaboration, literature search, writing first draft and editing. TS and MH provided literature searches, specifically those related to road traffic accidents in Pakistan. TS made the figures and tables. SS assisted with first draft of manuscript writing and editing. FM and SE provided literature feedback about brain injury numbers and databases that exist across various collaborating institutes and hospitals in Pakistan.

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REVIEW
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Subjective Cognitive Decline and Related Cognitive Deficits

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Since late stage dementia, including Alzheimer's disease (AD), cannot be reversed by any available drugs, there is increasing research interest in the preclinical stage of AD, i.e., subjective cognitive decline (SCD). SCD is characterized by self-perceptive cognitive decline but is difficult to detect using objective tests. At SCD stage, the cognitive deficits can be more easily reversed compared to that of mild cognitive impairment (MCI) and AD only if accurate diagnosis of SCD and early intervention can be developed. In this paper, we review the recent progress of SCD research including current assessment tools, biomarkers, neuroimaging, intervention and expected prognosis, and the potential relevance to traumatic brain injury (TBI)-induced cognitive deficits.

Keywords: subjective cognitive decline, mild cognitive impairment, Alzheimer's disease, traumatic brain injury, diagnosis, intervention

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BACKGROUND

In 1982, Reisberg (1) classified the course of Alzheimer's disease (AD) progression into the following seven stages according to the patients' clinical manifestations: (1) no cognitive decline, (2) very mild cognitive decline, (3) mild cognitive decline, (4) moderate cognitive decline, (5) moderately severe cognitive decline, (6) severe cognitive decline, and (7) very severe cognitive decline. The second stage was regarded as the earliest description of AD-related subjective cognitive decline (SCD). With the increased understanding of AD etiology detected by neuropathology, neuropsychology, pathophysiology, neuroimaging, and other techniques, the National Institute on Aging and Alzheimer's Association (NIA-AA) suggested that AD be subdivided into AD-preclinical stage, AD-MCI stage, and AD-dementia stage (2, 3). Of them, the preclinical phase of AD can be further divided into 3 substages: (1) amyloid deposition, (2) amyloid deposition and neuronal degeneration, and (3) neuronal degeneration and amyloid deposition with subtle cognitive decline (i.e., SCD stage). Until now SCD has had multiple names: subjective memory impairment (SMI), subjective cognitive complain (SCC), and subjective memory complaint (SMC) etc., with memory decline as the most prominent manifestation. With the recent research progress and understanding of the clinical symptoms of SCD from basic research and clinical studies, it is now recognized that visual space damage, language impairment, attention deficit, and other symptoms could co-exist in SCD patients. Therefore, "cognition" was the more appropriate word to describe the clinical characteristics of SCD rather than "memory" (4–6). Thus, the Subjective Cognitive Decline Initiative (SCD-I) Working Group has advocated the use of the name-SCD (6).

In 2014, the SCD-I working group proposed the SCD conceptual framework, and by redefining SCD, emphasized that the subjective perception of cognitive function decline of SCD patients is not a state and thus does not need to be confirmed by objective cognitive tests. Thus, the purpose of neuropsychological examination of SCD is to exclude the possibility of objective

cognitive impairment. SCD could be characterized as memory impairment or multiple cognitive domains impairment. The SCD-I conceptual framework also described important clinical features and accompanying symptoms of SCD, emphasizing that key information—such as age of onset, onset manifestation, symptom fluctuation, accompanying symptoms, circumstantial evidence, and comorbidity—should be collected in future research. The conceptual framework has also emphasized that the following features can increase the likelihood of preclinical AD in individuals with SCD: subjective perceptive memory decline, onset of disease within 5 years, onset at over 60 years of age, worrying about cognition decline, or subjectively feeling worse memory than the same age group. Specificity of the SCD diagnosis increases if the following conditions are present: informative confirmation, carrying ApoEe4 allele, and with positive AD pathophysiological biomarkers. In short, SCD has gradually become better understood.

Stewart and others (4, 7) have suggested that toward the end of the preclinical stage of AD, the subjective perception of cognitive decline in the elderly is more sensitive than objective neuropsychological tests. Pathological changes in AD could have occurred years ahead of the manifestation of MCI (8), which suggests that there should be a "pre-MCI" phase before the manifestation of MCI. The cognitive functional deficits of the patients could be reversible to normal at this stage. Thus, SCD may be a critical stage for early diagnosis and intervention of AD.

SCD is an intermediate state between normal cognition and MCI that may predict the development of objective cognitive decline (9–11). AD is a progressive disease and an abnormal deposition of Aβ and Tau in the brain may occur 15–20 years before the clinical diagnosis of AD, whereas MCI often occur 2 to 3 years before the onset of AD (12). Therefore, the extended SCD concept fills the gap between the manifestation of initial decline in cognitive function of AD and occurrence of MCI. The inclusion of the SCD concept completes the full picture of AD development (from normal state to SCD to MCI and to AD) and provides the basis for the diagnosis and intervention of AD at the very early stage of the disease (2, 6, 13).

EPIDEMIOLOGY

Epidemiological studies showed the prevalence of SCD ranged from 10 to 88%, with the highest prevalence found in the most advanced age group: 20% among people aged 65 years old and younger, 25 to 50% among people older than 65, and 88% among people older than 85 (4, 7, 14, 15). Jonker et al. (16) reported a 34.3% prevalence of SCD in community-based people aged 65–85 in the Netherlands, whereas Luck et al. (17) showed 12.3% prevalence of SCD in 117 of the 953 elderly participants (based on the general population sample). In a Spanish population-based cohort (ALFA project), 572 out of the 2670 participants studied reported SCD, showing a prevalence of 21.4% (18). Han et al. (19) explored the prevalence of SCD in the Shun Yi District in Beijing, China, by two standards. They found that the prevalence of SCD was 18.8% based on standard 1 (the Alzheimer's Disease Neuroimaging Initiative, ADNI) and 14.4% according to standard 2 (Jak/Bondi criterion), in their well-designed study. Another German study included 1,467 patients from clinics, wherein 792 (54.0%) reported SCD (20). In view of longitudinal studies, a multi-center longitudinal observational study (3-year follow-up) of the German Dementia Competence Network (DCN) revealed that 22% SCD patients progressed to MCI and 12% to AD, while only 3.7% subjects developed to a non-AD dementia (21). Some Swedish researchers found that in 122 SCD patients, over a follow-up period of 48 months, 39% declined cognitively and 10% converted to AD, in a clinical prospective single-center Gothenburg MCI study (22). Mitchell et al. (23) displayed that the conversion rate of SCD to MCI was 34.2% based on the community population over the mean period of 5 years through review of 28 studies. A recent 7-year follow-up study (24) showed that 212 subjects (109 patients with SCD) were considered: 26 out of 109 SCD subjects converted to MCI, 15 developed to AD, and 68 patients remained stable. A large-scale, 10-year-follow-up epidemiological survey conducted among 2,043 non dementia subjects showed that 372 old people developed dementia within 10 years (25, 26), of which 208 (55.9%) were diagnosed as AD. Cox regression analysis showed that SCD predicted all causes of dementia. From the results mentioned above, we can see that epidemiological datum of SCD differs from different studies because no common definition, criteria, and screening tools are available (27). Consequently, unified terminology and methodology and different populations (different age, community or clinic-based, etc.) are significant parts of further research.

CLINICAL CHARACTERISTICS

Subjective memory impairment (SMI), such as recent memory decline, is a common clinical manifestation of SCD. In comparison, only a few SCD patients reported subjective language impairment.

Emotional state and individual heterogeneities could affect SCD diagnosis. People with depression and anxiety tend to over worry about their cognitive ability, memory, or other cognitive decline (10, 25). These patients tend to regard normal physiological forgetfulness as severe memory decline and believe their cognitive functions are getting worse, increasing the physical and mental burden. And this kind of memory decline is often age-related, non-pathological, and without objective evidence. However, recent studies suggest that late-life depression is associated with increased risk of all causes of dementia, including AD (28, 29). Moreover, depression has been shown to be related to objective changes to brain structure and function (30), including gray matter abnormalities within frontal-subcortical and limbic networks (31) and loss of white matter integrity (32). Together, these findings suggest that arbitrary exclusion of persons with depression from SCD studies can result in incomplete understanding of the mechanisms by which SCD subjects progress to cognitive decline and dementia.

Furthermore, like depression and anxiety, enhanced stress levels and neuroticism may accelerate AD pathological progression and cognitive decline (33, 34). Therefore, it is often

difficult to make the clinical diagnosis or differential diagnosis of SCD for people with neuro-emotional comorbidity. They are more prone to be considered for an exclusionary diagnosis, in which comprehensive factors including age, gender, education, experience, emotional state, etc. are considered. Including such patients in SCD research would require quantification of the impact of depression and other psychiatric symptoms so that they can be adjusted as potential moderator variables (33, 34).

As mentioned above, SCD is characterized by self-experienced cognitive decline. Thus, informant confirmation has been speculated to increase the accuracy of diagnosis for preclinical AD spectrum. Many researchers also reckon that self- and informant-reports represent complementary approaches (33).

NEUROPSYCHOLOGICAL ASSESSMENT

Mini-Mental State Examination (MMSE) and Montreal Cognitive Assessment (MoCA) are two widely used screening tools that evaluate overall cognitive function, with high sensitivity for detecting MCI and AD. However, for detection and assessment of SCD, their validations or alternatives are needed. Sahlgrenska Academy Self-Reposed Cognitive Impairment Questionnaire (SASCI-Q) has been used to distinguish SCD from normal cognitive population and to evaluate daily cognitive ability in several studies (37). The Memory Alteration Test (M@T) has been reported to have distinguished SCD from amnestic mild cognitive impairment (aMCI) and early AD with higher sensitivity and specificity in comparison with MMSE (37). However, M@T is not a comprehensive tool for evaluating the overall level of cognition (38, 39). The Subjective Cognitive Decline Questionnaire (SCD-Q) is a more informative test that can distinguish SCD patients from the normal cognition population (37, 40). The test places an emphasis on the concept of "cognitive-decline-as-chief-complaint in different sources." The complementary confirmation of cognitive decline by both subjective and objective perception makes The Subjective Cognitive Decline Questionnaire (SCD-Q) a great screening tool in the diagnosis and prognosis of SCD (41). Other neuropsychological examination methods evaluated by different study groups include the Memory Complaint Questionnaire (MAC-Q) and Everyday Cognition (ECog) (42, 43). The most common cognitive impairment of SCD is episodic memory, followed by executive function. Researchers tried to screen SCD patients from elderly people with normal cognition using the Stroop Color Word Test (CWT) and found significant differences in the outcomes between the SCD and normal cognition individuals by using the CWT rather than general neuropsychological tests, which indicated that CWT could be used as an independent indicator of SCD in clinical management (44). Recently, Ismail et al. (45) developed the mild behavioral impairment checklist (MBI-C) questionnaire for SCD and MCI, with the aim to develop more accurate assessment tools for screening the clinical manifestations of patients at early-stage-AD. Some recent research indicated that neuropsychological assessment may aid clinical diagnosis, especially memory neuropsychological tests which may also be useful for evaluating the risk of progression to AD among SCD subjects (24, 46). However, most scales and evaluation methods are still in the development stage or in their initial testing stage. More large-scale, multi-center, and standardized studies are needed to determine the suitability for clinical application as well as unified screening tools.

BIOMARKERS

Valid SCD biomarkers may reflect the intermediate stage between clinically normal older adults (yet still with potential SCD) and MCI individuals, which is in line with how SCD is currently conceptualized. Any established associations between SCD and biomarkers would further validate SCD as a valid stage prior to the onset of clinical impairment along the early AD trajectory (33). In this section, biomarkers of cerebrospinal fluid (CSF) are emphatically introduced.

Biomarkers of CSF are also significant auxiliary diagnoses and predictors of SCD. Biomarkers in the CSF of SCD patients may occur well before any significant changes in brain MRI. The low level of Aβ protein in CSF indicates increased amyloid protein deposition in the brain, while high levels of T-tau and P-tau proteins suggest neurodegeneration. Leoni et al. (47) reported that as the cognitive function declined, T-tau and P-tau proteins in CSF of SCD patients increased significantly whereas Aβ protein decreased significantly. These findings are consistent with the 2011-National Institute on Aging and Alzheimer's Association (NIA-AA) diagnosis standard for SCD (48). Although changes in Aβ protein and tau protein in the CSF of SCD population could be a possible diagnostic basis for SCD, other potential independent causative factors, such as severe anxiety and depression, should be excluded. A 5-year follow-up study of 149 patients (94 MCI patients, 55 SCD patients) conducted by Sierra-Rio et al. (49) showed that during the follow-up period, the ratio of CSFAβ42/P-tau decreased in 72.4% of the patients (MCI83%, SCD27%). Because all of these patients progressed to AD eventually, these results suggest that the decreased Aβ42/P-tau ratio in CSF could be a critical risk factor for SCD/MCI progressing to AD. Compared to tau protein or other related predictors, Aβ42 could better predict the progression of SCD to MCI or to AD as proposed by Van Harten et al. (50). They suggested that Aβ42 level is a better predictor for clinical progression of SCD whereas T-tau is a predictor for MCI progression. Another cohort study conducted in Holland by the same research group found that SCD patients with initial abnormal CSF levels of Aβ42, T-tau, and P-tau have increased the risk of progression into MCI or AD within 2 years by 16, 2.8, and 2.6 times, respectively, compared to those with normal levels, suggesting that low level Aβ42 is a strong predictive factor for cognitive decline in SCD patients (50). Indeed, patients with detectable biomarkers are more likely to undergo cognitive decline during the course of SCD progression.

Jia et al. (51) found that the exosomal concentrations of Ab42, T-tau, and P-T181-tau in AD patients were higher than those in aMCI and control groups and the level of each exosomal biomarker was highly correlated with that in CSF.

Their multicenter study with two-stage design verified the consistence between CSF and blood exosomal biomarkers and confirmed that exosomal Ab42, T-tau, and P-T181-tau have the same capacity as those in CSF for the diagnosis of AD and aMCI. This kind of peripheral blood will play a significant role in non-invasive detection at the early stage of AD. However, these findings, including CSF and peripheral blood, still need further confirmation in longitudinal studies.

NEUROIMAGING EXAMINATION

Many neuroimaging methods have been applied to diagnose SCD. Among them, Structural MRI (sMRI) can be applied to measure the volume and thickness of the patients' cortex (38). There is evidence to show that at SCD stage, the degree of the cortex atrophy is associated with the severity of cognitive impairment. Saykin et al. (52) reported that, compared to the normal population, the level of medial temporal lobes atrophy and frontal lobes atrophy of SCD patients is related to the degree of cognitive decline. Recent MRI studies also showed that people with MCI differed from very mild cognitive decline and normal controls in the right hippocampus volume (53, 54). People with low-volume right hippocampus and cognitive impairment are at greater risk of advancing to AD (53, 54). Overall, sMRI is expected to be a useful tool for diagnosis of SCD.

Functional MRI (fMRI) studies also show a weakened resting state in some of the brain regions of AD patients, such as the right hippocampus and insular subregions, indicating damage in these regions. Whereas, the increased activities in certain brain regions may suggest functional compensation in these areas at the early stage of AD (see **Figure 1**). Regarding SCD, the fMRI study by Ying's groups showed that (55), compared to the control group,

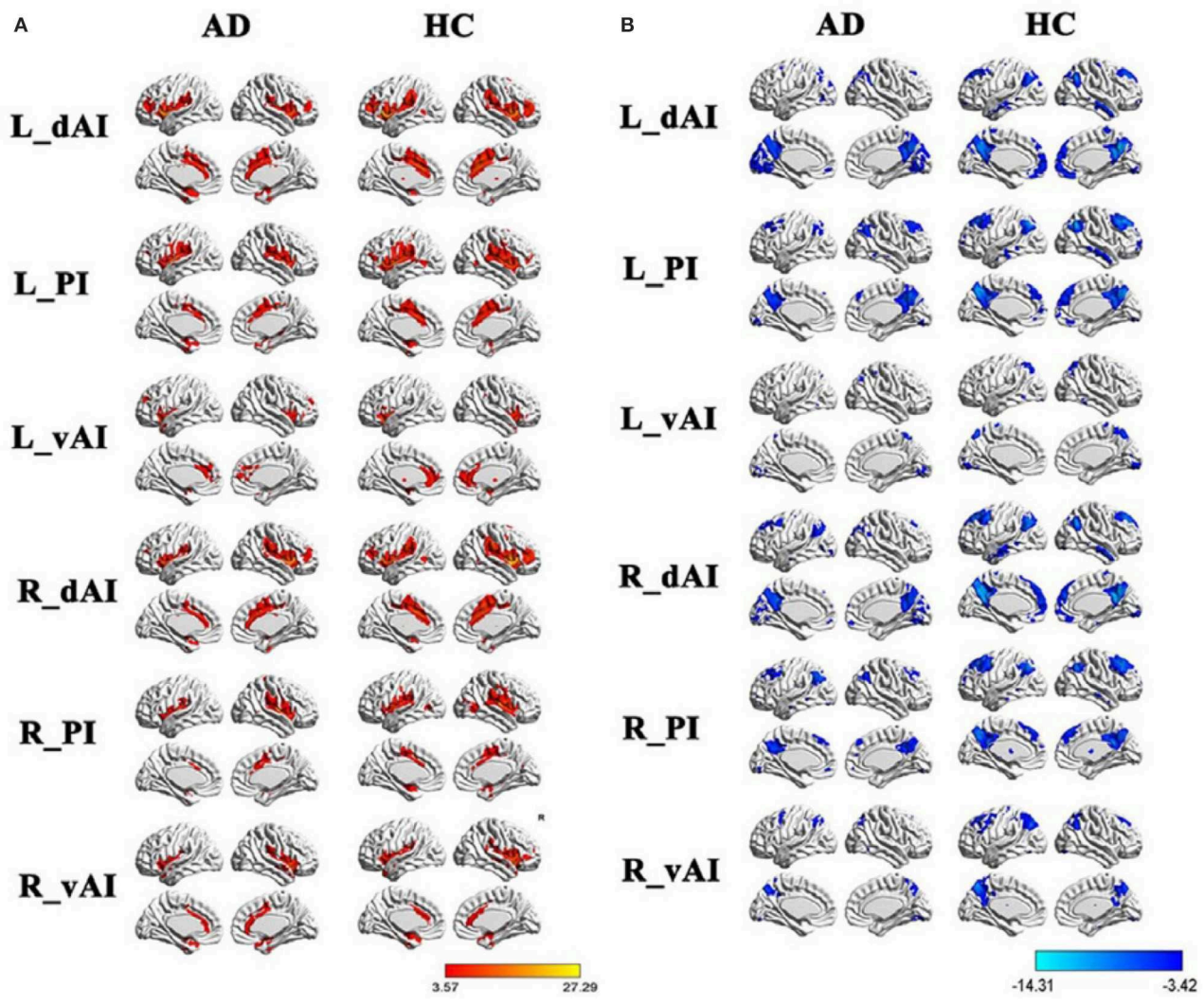

FIGURE 1 | Within group resting-state functional connectivity (RSFC) analysis. **(A)** The positive RSFC patterns of the insular subregions in each group including healthy controls and AD patients. **(B)** The negative RSFC patterns of the insular subregions in each group including healthy controls and AD patients. PI, posterior insula; dAI, the dorsal anterior insula; vAI, the ventral anterior insula. Figure is reproduced from He et al. (35), with authors permission.

there was a significant increase in the activity of the resting state in the following brain regions of SCD patients: inferior parietal lobule, right superior temporal gyrus, right inferior temporal gyrus, fusiform gyrus, and right posterior lobe of the cerebellum. This confirms the existence of compensatory mechanisms in spontaneous activities of SCD patients at the resting state. Another study suggested that patients with APOEε4 presented both increased and decreased functional connectivity as reflected in default mode network (DMN), which is correlated with clinical cognitive performances based on the regions of interest (ROI)-based functional connectivity analyses and voxel-based analyses (56). Thus, altered functional connectivity may be an early sign of cognitive decline. A resting-state fMRI study mainly revealed that SCD individuals had reduced correlations between centrality frequency (i.e., across the entire time window, the proportion of time that the hub appeared was defined as centrality frequency) of the anterior cortical regions and cognitive performance, compared with normal controls (NCs). In contrast, correlations between centrality frequency of the posterior cortical regions and cognitive performance were stronger in SCD participants than NCs. Besides, the alterations mainly concentrated on the anterior and posterior regions associated with the default mode network (DMN) (36). See **Figure 2**.

Glucose is an important energy source for brain function. Abnormal glucose uptake in different regions may be related to the extent of altered brain function. 18F-fluorodeoxyglucose position emission tomography (FDG-PET) can reflect changes in glucose metabolism in different parts of the brain (57). Scheef et al. (57) reported that, compared to the control group, metabolic rates in right precuneus and left parietal cortex are lower in SCD patients, while metabolic rates in medial temporal lobe and right para-hippocampal gyrus are higher, suggesting simultaneous abnormal energy metabolism and neuronal dysfunction in certain regions of SCD patients. And the decline in executive ability of SCD patients was associated with Aβ deposition in a Pittsburgh Compound B PET (PIB-PET) study (58). In recent years, breakthroughs in SCD research have been achieved by applying Flobetapir F-18 to Florbetapir-PET (amyloid-PET) techniques with improved sensitivity. Flobetapir F-18 has a higher affinity for Aβ accumulated in the brain and the

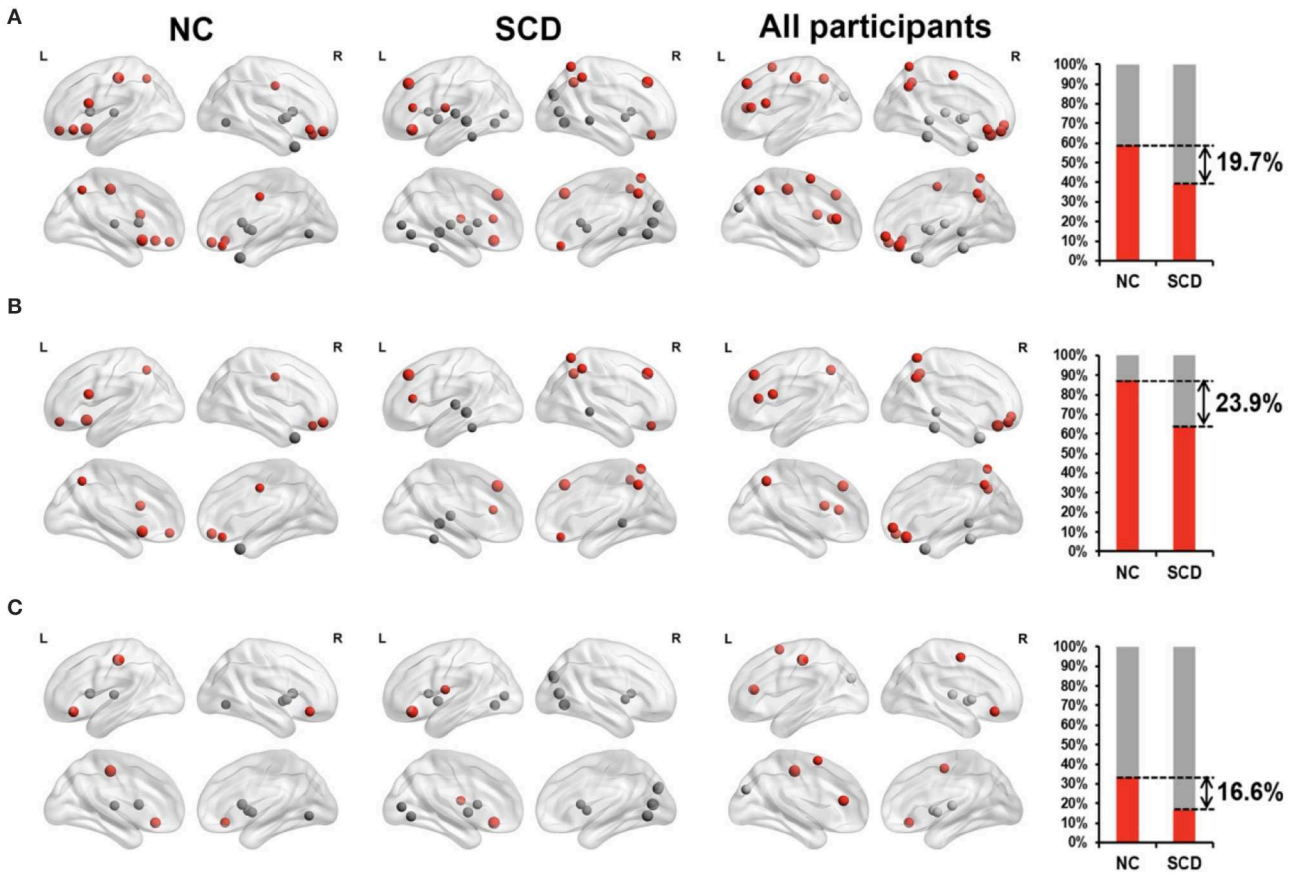

FIGURE 2 | Relationship between degree of centrality (reflecting the dynamic functional connectivity) and neuropsychological tests in NC individuals (the first 2-columns), SCD patients (the second 2-columns), and all participants (the third 2-columns). The far-right column displayed the percentage of significantly correlated regions ($p < 0.05$) located in anterior (red) cortical and other (gray) cortical regions in the two groups. The nodes covered by red are located in anterior cortical regions while nodes covered by gray are located in posterior cortical regions or subcortical regions. **(A)** All the significant correlated regions ($p < 0.05$). **(B)** The regions in the DMN. **(C)** The regions out of the DMN. Figure is reproduced from Han et al. (36), with authors permission.

results of Florbetapir-PET are highly consistent with that of Aβ in CSF that may be used as an early clinical diagnosis (59). Clark et al. (60), found an association between amyloid -PET imaging and the density of brain β amyloid protein. These findings show that PET can be an effective tool for detecting abnormalities in brains at SCD stage from function and Aβ deposit perspective.

Mallio et al. (61) demonstrated an epicentral disruption of structural connectivity in aMCI and AD around entorhinal and hippocampal regions, using diffusion-weighted imaging (DWI) consistent with the transneuronal spread hypothesis. Hereafter, researchers gradually paid attention to investigating brain network by Magnetic Resonance Diffusion Tensor Imaging (MR-DTI), and found that structural network properties might be preserved in patients with SCD but disrupted in aMCI stage, which may contribute to a better understanding of pathological mechanisms of AD (62). Li et al. (63) analyzed the DTI data in SCD patients by using Tract Based Spatial Statistics (TBSS), and found extensive white matter damage in SCD patients. These studies suggested that these pathological changes in SCD subjects were undetectable by conventional tests. Han et al. (64) also used DTI and graph theory approaches to demonstrate disrupted topologic efficiency of the brains' structural connectome of SCD. Thus, connectome-based biomarkers could be potentially used for detection of SCD in an elderly population. Another DTI study (65) revealed that rich club organization (some certain cortical regions highly connected to each other, with other regions referred to as peripheral) was destroyed, with less structural connectivity among rich club nodes in persons with MCI or AD but remained stable in SCD patients, which can show the development of AD and be viewed as a biomarker for early diagnosis. A recent systematic review including 16 studies (using neuroimaging tools containing Magnetoencephalography, structural and functional MRI, DTI, etc.) concluded that patients with SCD exhibited a significant abnormality of the brain network, compared to healthy controls, which was damaged in a similar approach as in Mild Cognitive Impairment (66). Magnetic Resonance Diffusion Kurtosis Imaging (DKI) is a relatively new technique that is an extension of DTI and is based on the non-Gauss-field of the water molecular diffusion. DKI can solve the problem of nerve-fibers-crossing and is more sensitive in observing subtle changes in brain white matter and gray matter (36, 56). Although only a few SCD studies reported using DKI, DKI will play an increasingly important role in the study of SCD in the future.

Together, comprehensive application of neuroimaging technology provides a valid way of capturing early brain alterations, and as such, these approaches may complement the absence of other neuropsychological tests and CSF biomarkers available for SCD diagnosis.

PROGNOSIS OF SCD

The development of SCD could follow any of these three directions: (1) remission and symptoms fade away, (2) stabilized, (3) become worse and progress to MCI or AD. The conversion rate of SCD to MCI and AD in diagnosed SCD was reported to be 54.2%, of which 78.9% progressed to MCI in a 7-year follow-up study (4). In comparison, the risk of developing MCI or AD in the normal group was 14.9%, of which 71.4% developed to MCI. After adjusting for confounding factors, the risk for SCD group to develop to MCI or AD within 7 years was 4.5 times greater than that of no-SCD group. A meta-analysis by Mitchell et al. (23), showed that the risk of progression to AD in the elderly with SCD was 2 times greater than the elderly without SCD, with the annual conversion rate of SCD to MCI and AD as 6.6 and 2.3%, respectively. Importantly, many studies also suggested that low CSF Aβ42 and high t-tau or p-tau may predict cognitive decline (progression to MCI) in SCD subjects (67).

INTERVENTION

So far there is no FDA-approved pharmacologic interventions for SCD. A few supplements-based interventions have been reported. A 12-week dietary supplements treatment was conducted among 30 SCD patients (68), using active ingredients including Vinpocetine,uridine-5 monophosphate, hops, ginger, rosemary, Ashwaganda, grape seed, wild blueberry extract, L-alpha, Omega-glycerophosphate choline-3 phosphatidylserine, etc. The results showed significantly improved cognitive function in all subjects at the second week assessment, but no further improvement was found at the end of the study. Another report by Williams et al. (69) proposed that cognition training, vegetable intake, Mediterranean diet, Omega-3 fatty acid, physical activity, and non-physical leisure activities, when combined, could prevent cognitive decline. Among various nutritional markers related to aging and cognition, experts found that there may be an association between low-level Vitamin D and likelihood of functional deficits, such as coexisting or separate physical and cognitive decline in patients with subjective memory complaints (70). Researchers also found that a 4-week simultaneous memory training and aerobic exercise program can contribute to improving memory, reasoning abilities, and attention in a recent study (71). At the 2017 London Alzheimer's Association International Conference (AAIC), "Lancet" International Disease Prevention, Intervention and Nursing Committee released a new analysis system that identified nine modifiable risk factors, including <12 years of education, hypertension, obesity, hearing loss, depression, diabetes, lack of exercise, smoking, and social isolation. Most of these risk factors could be modified from childhood to middle age and account for 35% of AD. Using the stratified cluster random sampling method, Han et al. (19) investigated the prevalence and risk factors of SCD in Shun-Yi District of Beijing, China, and found high prevalence of SCD among people who were of an old age, had a low education, less social support, and daily drinking habits, highlighting these as independent risk factors. Because most of these factors are modifiable except age, the preservation and mobilization of brain plasticity by cognitive training, healthy diet, active aerobic exercise, smoking cessation, alcohol restriction, control of diseases such as diabetes, hypertension, dyslipidemia, anxiety and depression, enriched social activities, alone or combined, could be potential directions of intervention.

TABLE 1 | Summary of recent TBI studies with cognitive decline.

References, Country	Design	(1) Injury severity (2) Diagnosis method	Assessment times (post injury)	Assessment tool	Conclusion
Chen et al. (90) China	Prospective phase II pilot study	(1) Mild TBI (2) not reported	Baseline: 1 day t1: 7 ± 2 days t2: 28 ± 4 days t3: 84 ± 10 days	MMSE, Cognitive Abilities Screening Instrument	Cerebrolysin improved the cognitive function of patients with mild TBI at the third month after injury
Covassin et al. (91) USA	Prospective cohort	(1) sport-related concussion (2) American Academy of Neurology graded concussion assessment;	Baseline: preseason t1: 2 days t2: 7 days t3: 14 days	Post-Concussion Assessment and Cognitive Testing	The outcomes supported sex differences in memory and symptoms, age differences in memory and an interaction between sex and age on postural stability after concussion
Dikmen et al. (92) USA	Prospective longitudinal	(1) Mild TBI (2) GCS, CT	Baseline: 1 month t1: 12 months	Wechsler Adult Intelligence Scale, Halstead-Reitan neuropsychological battery, Simple Reaction Time Test, Finger-Tapping Test	Although the majority of neuropsychological and functional differences abated by 1 year, reporting three or more post-traumatic symptoms remained for around 50% of individuals
Failla et al. (93) USA	Prospective cohort	(1) Severe (2) GCS, GOS	Baseline: 6 months t1: 12 months	Functional Independence Measure-Cognition, Trail Making Test A and B, Digit Span test, Rey-Osterreith Complex Figure Test, II California Verbal Learning Test (Edition 2), Delis-Kaplan Executive Function System, Stroop, Controlled Oral Word Assoc Test	The results revealed that genetic variation within DRD2 was associated with cognition recovery post TBI
Farbota et al. (94) USA	Prospective cohort	(1) Moderate to severe (2) GCS, MRI	Baseline: 2 months t1: 12 months t2: 4 years	Controlled Oral Word Assoc Test, Wide Range Achievement Test, Reading Subtest (Edition 3), Wechsler Adult Intelligence Scale (Edition 3), Trail Making Test A and B	The data showing progressive white matter damage for several years after TBI supported the hypothesis that TBI should be regarded not as an isolated incident but as a prolonged disease state
Kontos et al. (95) USA	Prospective longitudinal	(1) Mild TBI (2) GCS	Baseline: pre-injury t1: 1–7 days t2: 8–20 days	Military Immediate Post-Concussion Assessment and Cognitive Testing	Decreases in neurocognitive performance and increased mild TBI symptoms were observed in the first 1 day to 7 days after combat-related mild TBI, and a history of blast-related mild TBI may influence these effects
Meier et al. (96) USA	Prospective longitudinal	(1) sport-related concussion (2) MRI	Baseline: 1.41 ±0.94 days t1: 8.73 ± 2.19 days t2: 31.46 ± 4.67 days	Automated Neuropsychological Assessment Metrics	The outcomes showed the evidence of reduced cerebral blood flow in human concussion and subsequent recovery, which may predict consequences following concussion
Ponsford et al. (97) Australia	Prospective longitudinal	(1) Mild TBI (2) GCS	Baseline: 0–48 h t1: 1 week t2: 3 months	Immediate Post-Concussion Assessment and Cognitive Testing	Patients with mild TBI were more likely to report ongoing memory and concentration problems in daily activities after trauma recovery
Register-Mihalik et al. (98) USA	Prospective longitudinal	(1) sport-related concussion (2) GCS	Baseline: preseason t1: 2.36 ± 1.41 days	Automated Neuropsychological Assessment Metrics	The data suggested that the multifaceted battery is more sensitive than any single measure in clinical concussion measures, and sensitivity to balance and neurocognitive impairments was low for each individual measure
Robertson and Schmitter-Edgecombe (99) USA	Prospective longitudinal	(1) Moderate-severe (2) GCS, post-traumatic amnesia	Baseline: 45.00 ± 35.14 days t1: 280.11 ± 104.11 days	Symbol Digit Modalities Test, Trail Making Test, Rey Auditory Verbal Learning Task, Wechsler Adult Intelligence Scale, Controlled Oral Word Assoc Test	Outcomes showed that the error-monitoring performance of patients with TBI was significantly poorer than controls at both baseline and follow-up
Sandhaug et al. (100) Norway	Prospective cohort	(1) Moderate, severe (2) GCS, loss of consciousness	Baseline: 3 months t1: 12 months t2: 25 months	Functional Independence Measure-Cognition, Glasgow Outcome Scale Extended	Functional Independence Measure and Glasgow Outcome Scale Extended in TBI research were more relevant for assessment of the functional recovery in a sub-acute phase than in later stages of TBI recovery

(Continued)

TABLE 1 | Continued

References, Country	Design	(1) Injury severity (2) Diagnosis method	Assessment times (post injury)	Assessment tool	Conclusion
Schmitter-Edgecombe et al. (101) USA	Prospective longitudinal	(1) Moderate, severe (2) GCS, post-traumatic amnesia	Baseline: 41.85 ± 25.68 days t1: 289.00 ± 85 days	Rey Auditory Verbal Learning Task, Controlled Oral Word Assoc Test, Wechsler Adult Intelligence Scale, Letter Number Sequencing Test, 5-point test, self-ordered pointing test, Trail Making Test	TBI patients showed recovery in both content and temporal order memory for activities during the first year, however, activity memory performances remained poorer than controls at follow-up. Greater self- and informant report of everyday memory difficulties was relevant to poorer temporal order memory
Sours et al. (102) USA	Prospective longitudinal	(1) Mild TBI (2) GCS, CT	Baseline: 7.7 ± 2.4 days t1: 36.0 ± 8.2 days	Automated Neuropsychological Assessment Metrics	The results exhibited that reduced interhemispheric functional connectivity may result in the subtle cognitive deficits in mild TBI patients with serious symptoms
Vanderploeg et al. (103) USA	Prospective longitudinal	(1) Moderate to severe (2) post-traumatic amnesia, loss of consciousness	Baseline: 32.4 ± 12.8 days t1: 6 months t2: 12 months	California Verbal Learning Test	The results were in support of an impaired consolidation hypothesis as the primary deficit underlying memory impairment in TBI
Veeramuthu et al. (104) Malaysia	Prospective longitudinal	(1) Mild TBI (2) post-traumatic amnesia, loss of consciousness, CT, GCS	Baseline: 4.4 ± 8.3 h after gain of consciousness t1: 6 months	Neuropsychological Assessment Battery-Screening Module	The uncomplicated mild TBI group exhibited slower recovery, especially in tasks of memory, visuospatial processing, and executive functions, at follow-up, compared with patients with complicated mild TBI
Wang et al. (105) China	Prospective longitudinal	(1) Moderate to severe (2) GCS, CT, MRI	Baseline: 5.86 ± 4.54 years t1: 6 months following baseline	Functional Independence Measure-Cognition	The results demonstrated that the umbilical cord mesenchymal stem cell transplantation improved the neurological function and self-care in patients with TBI
Wylie et al. (106) USA	Prospective cohort	(1) Mild TBI (2) GCS, CT, loss of consciousness	Baseline: 2.0 ±0.9 days t1: 8.7 ± 1.2 days	Immediate Post-Concussion Assessment and Cognitive Testing	The data provided neuroimaging evidence for working memory deficits during the first week following mild TBI. Patients with persistent cognitive symptoms after mild TBI had increased requirement for posterior cingulate activation to finish memory tasks at 1 week following a head trauma
Zaninotto et al. (107) Brazil	Prospective longitudinal	(1) Moderate to severe (2) GCS, MRI	Baseline: 6 months t1: 12 months	Rey-Osterreith Complex Figure Test, Wechsler Adult Intelligence Scale (Edition 3), Grooved Pegboard Ask	The use of citicoline for 3 months, compared with placebo, did not result in enhancement in functional and cognitive status

Recently, numerous systematic reviews and meta-analyses (72) indicate that non-pharmacologic intervention (NPI) can be an effective intervention for SCD, particularly, cognitive interventions can benefit objective cognitive functioning, offset some normal age-related cognitive decline, support productive aging, and enhance quality of life for individuals who may otherwise develop MCI or dementia, such as mind-body exercise, especially Tai Chi, a well-known type of traditional Chinese martial arts (73). Some researchers also found that ketogenic diet may be another early non-pharmacologic intervention in AD (74). NPI could be more useful than medication due to its cost-effectiveness, lack of side effects, the fact that it can be readily adapted by a wide diversity of appropriately trained and experienced health professionals (72), and it is administrable before symptom onset (75). However, there is still a lack of high-quality research (such as randomization, blinding of participants and clinicians, use of "intention to treat" analysis for incomplete outcome data, etc.) in this direction (76).

RELEVANCE TO TRAUMATIC BRAIN INJURY-INDUCED COGNITIVE DEFICITS

The past decades have seen traumatic brain injury (TBI) rush into the forefront of neurology due to the increasing incidence of falls, motor vehicle accidents, sports injuries, and wars (77). TBI is defined as altered brain function or other evidence of brain pathology caused by an external force (jolt to the head, blow, or other such penetrating head injury) and, more importantly, has been increasingly recognized as a risk factor for cognitive decline, dementia, and AD (78). Numerous studies also showed that there was a linear relationship between TBI severity and cognitive consequences after TBI, more specifically, the greater the severity, the greater the outcomes. To date, some links and details of mechanisms by which TBI leads to cognitive impairment remains to be fully elucidated. However, it is believed that excitotoxicity mediated by an overproduction of excitatory neurotransmitters like glutamate extracellularly, is hypothesized to be the sentinel event after TBI (79). Neuroinflammation is

also activated in response to TBI, and has both beneficial and hazardous consequences. Some of the released pro-inflammatory cytokines, anti-inflammatory cytokines, and chemokines may induce Aβ plaque deposition in the brain (80, 81). After TBI, some brain areas have been shown to suffer from hypoxic damage and secondary ischemia (82), which is involved in the pathogenesis of AD by accelerating the accumulation of Aβ and increasing the hyper-phosphorylation of tau, resulting in chronic neurodegeneration (82). Furthermore, a growing number of studies have focused on the cerebrovascular link between TBI and AD. Cerebrovascular outcomes of TBI include edema, hemorrhages, vasospasms, changes in cerebral blood flow (CBF), blood-brain barrier (BBB) breakdown, coagulopathy, and chronic inflammation (83). TBI is a trigger of neurodegeneration and is a useful model for studying certain pathological features of AD, such as Aβand tau deposition. Conversely, Aβ and tau release can lead to cerebrovascular injury, and their accumulation around cerebral micro-vessels has a deleterious chronic impact (84, 85). In addition, pericyte dysfunction and alterations in endothelial cell after TBI are contributors to the neuropathology and cognitive deficits. Indeed, TBI is intimately related to cognitive decline.

As mentioned above, patients who report a history of TBI are more likely to precede an onset of AD and enough data support some overlapping distributions of pathology between them. In each of these two disorders, the final common pathway into clinical symptomatology includes the malfunctioning and death of neurons. Therefore, it is considered that, for AD investigators, it might be better to pay attention to different research directions that potentially offer opportunities to outflank TBI rather than frontally assault, AD, because of failure of related clinical trials before, in more depth, TBI as a possible model could contribute to better understanding the puzzle of AD. Specifically, a TBI provides investigators with the opportunity to induce many of the shared neuropathologies by animal models for TBIs or study them in patients who have suffered a head injury. TBI also offers basic and clinical investigators a temporally condensed microenvironment potentially reflecting within days and weeks of neuropathological progressions that can be studied in AD animal models or patients only by cross-sectional sampling of at risk subjects chosen from a population spread across decades (78). And we speculate that some neuropathologies may be observed at early stages of cognitive impairment because head injuries can be viewed as a starting point. Also, we are supposed to focus on prevention at the early stage, SCD, due to disappointing outcomes of AD therapy (78, 86, 87). Thus, TBI may be a definite breakthrough-point for studies of SCD. Additionally, it is noteworthy that subjective cognitive decline complaints after TBI and post-traumatic stress disorder (PTSD) have frequently been reported (88, 89), however, high anxiety and low mood resulting from TBI and PTSD may significantly influence subjective cognitive function, especially subjective appraisal of memory without any objective decline. Thus, clinicians should take that into account when distinguishing between TBI-induced affective disorders (anxiety, depression, etc.) and AD-related SCD, especially in old patients (89). Overall, there are differences and interrelations between TBI-induced SCD and AD-related SCD, and TBI is a significant focus of dementia research, with both basic and clinical research.

A summary of recent studies of TBI and cognitive decline is listed in **Table 1**.

TABLE 2 | Summary of main findings or conclusion for each section.

SCD	Main findings or conclusion
Concept proposing	AD-related SCD is an intermediate state between normal cognition and MCI that may predict the development of objective cognitive decline
Epidemiology	Epidemiological datum of SCD differ from different studies because of lack of unified terminology, criteria, screening tools and so on
Clinical Characteristics	Memory decline is a common clinical manifestation (excluding depression, anxiety and so on)
Neuropsychological Assessment	Most neuropsychological scales are still only for research
Fluid biomarkers	CSF and peripheral blood are meaningful predictive biomarkers for SCD
Neuroimaging Examination	Comprehensive application of neuroimaging technology (PET, fMRI, DTI, etc.) contribute to the diagnosis of SCD and better understanding the pathology
Prognosis of SCD	Remission, stabilized and progressing to MCI or AD
Intervention	NPI can be effective intervention for SCD
Relevance to Traumatic Brain Injury-induced cognitive deficits	1. Although TBI is a potential risk factor of AD and affective disorders, currently, it is not clear how TBI-related cognitive deficits are associated with SCD and AD. 2. SCD is often observed in patients with a history of TBI. 3. People with TBI and AD also share similar pathological alterations. 4. If a relationship between TBI (-related SCD) and dementia can be established, better diagnosis and early prevention strategy could be designed to prevent TBI-related SCD and dementia

CONCLUSION

The concept of SCD could shift the ineffective late-stage diagnosis and ineffective treatment of AD into a more effective prophylactic strategy. To better explore the field of preclinical AD, several significant issues should be resolved. First, standard terminology and assessment practices should be unified and adopted. Second, due to SCD's heterogeneity and subjectivity, comprehensive assessment methods can enhance the predictive validity of SCD as a marker for preclinical AD, including neuroimaging, CSF, blood, informants' complaints, and so on. Third, intervention trail, especially well-designed NPI for patients with SCD, is still a hotspot. Last but not least, studies of TBI animal models or patients may contribute to better understanding of more complex mechanisms of SCD, resulting from many neuropathologies similar to AD. Consequently, large-cohort, multi-centered, and longitudinal studies are needed to achieve more breakthroughs in the field of cognitive impairment.

A summary of the main findings or conclusions for each section is listed in **Table 2**.

AUTHOR CONTRIBUTIONS

TS wrote the first draft of this manuscript. YH and GX critically revised the manuscript. All authors read and approved the final manuscript.

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frontiers in Neurology

BRIEF RESEARCH REPORT
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Repeated Long-Term Sub-concussion Impacts Induce Motor Dysfunction in Rats: A Potential Rodent Model

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Whilst detrimental effects of repeated sub-concussive impacts on neurophysiological and behavioral function are increasingly reported, the underlying mechanisms are largely unknown. Here, we report that repeated sub-concussion with a light weight drop (25 g) in wild-type PVG rats for 2 weeks does not induce detectable neuromotor dysfunction assessed by beamwalk and rotarod tests. However, after 12 weeks of repeated sub-concussion, the rats exhibited moderate neuromotor dysfunction. This is the first study to demonstrate development of neuromotor dysfunction following multiple long-term sub-concussive impacts in rats. The outcomes may offer significant opportunity for future studies to understand the mechanisms of sub-concussion-induced neuropsychological changes.

Keywords: beamwalk, mild traumatic brain injury, neuromotor function, rotarod, sub-concussion

INTRODUCTION

Non-concussion or "sub-concussive" head impact is defined as "a cranial impact that does not result in known or diagnosed concussion on clinical grounds, which may be the result of a slosh phenomenon" (1). However, an increasing number of clinical studies report that when the sub-concussion is repeated over a long-term, it results in substantial neurological and neuropsychological alterations (1). For instance, studies in soccer players demonstrated that the frequency of ball headings correlated with structural changes of the brain (2, 3). However, very few studies to date investigated the effects of repeated sub-concussion in animal models (4), and its underlying mechanisms remain largely unknown. One of those studies by Shultz et al. showed that a single sub-concussion impact by mild lateral fluid percussion injury (0.50–0.99 atm) resulted in no neurobehavioral changes, whilst elevated neuroinflammation was evident (5). Although the mechanical force of sub-concussion and duration of sub-concussive impacts in rats are not directly

translatable to humans, this study presented no behavioral or clinical changes in the rats, falling into the aforementioned definition of sub-concussion. Furthermore, a study by Xu et al. used a weight-drop model to compare the effects of sub-concussion induced by different weights of 20, 40, and 60 g (6). The study found that the increasing weight corresponded with increasingly severe traumatic axonal injury in optic nerves, corpus callosum and cerebellum, without showing significant motor deficits. However, these studies only used a single sub-concussive impact, and do not represent frequently repeated sub-concussive impacts over a prolonged period of time.

Thus, in the present study, we employed lighter sub-concussive impacts with a 25 g weight-drop in wild-type PVG rats, which was 10-fold lighter than our previous study where more severe form of concussion was demonstrated (7). The weight drop was repeated multiple times (10 impacts/d, 3 d/week) for an extended period (2 or 12 weeks). The weight of 25 g was chosen because the previous study by Xu et al. demonstrated that 20–60 g weight drop provides sub-concussion-like impacts in rats (6). In addition, our pilot studies found that unlike the previously described procedure with 250 g weight (7), the rats receiving 25 g weight drop did not fall through the hole in the stage of the weight-drop device to create body rotation movement, thus the body and head remained on a holding paper towel. The latter is ideal for representing minor head knocks with minimum or no spinal movement/whiplash injury, which are the lighter end of sports-associated concussive impacts. Due to its substantial differences in biology and physiology, the frequency and duration of rat's sub-concussion procedure are not directly translatable to human. Nonetheless, a previous study by Agoston reports that based on longevity differences, one rat day is approximately equivalent to 27 human days, whilst other measures such as metabolic rate, protein turnover and heart rate present significantly different timelines between rats and humans (8). Moreover, the paper indicates that such timeline can substantially vary depending on the life stage (e.g., adulthood vs. adolescence). For simplicity, we used the longevity based equation to estimate an approximate human equivalent duration and frequency of our sub-concussion procedure as summarized in **Table 1**.

Thereafter, we examined the effects of the long-term repeated sub-concussive impacts on neuromotor performance and tested whether the duration of the sub-concussion period impacted on any significant differences.

MATERIALS AND METHODS

Animals

A total of 40 female PVG rats at 5 to 6 weeks old were purchased from Animal Resources Centre (WA, Australia) and were randomly allocated to sub-concussion group (SC) or Sham group. Female rats were selected to be consistent with our previous studies of concussion (7, 9). Young rats were chosen to be consistent with clinical findings, where soccer ball headings in childhood appear to induce neuronal impairment (3). The rats were maintained on standard maintenance chow purchased from Specialty Feeds (WA, Australia, Meat Free Rat and Mouse Diet). All animal procedures in this study were approved by the Curtin University Animal Ethics Committee (ARE2019-13/19).

Sub-concussion and Sham Procedure

Sub-concussive impacts were administered to the SC group rats with a custom-built weight-drop device (Northeast Biomedical, MA, USA) as described previously with minor modification (weight changed from 250 to 25 g) (7). Briefly, a 25 g weight was dropped from 1 m height to the impact site on lambda (2–3 mm anterior to the front of ears) under 3% isoflurane anesthesia. The weight drop was administered 10 times consecutively, with ∼10–30 s between each impact and after each impact, the impact site was re-aligned to lambda. The series of 10 SC impacts was repeated 3 days per week (i.e., Monday/Wednesday/Friday) for a period of 2 or 12 weeks. Sham group rats received 3% isoflurane without the weight drop for the same duration as SC procedure. After the SC or Sham procedure, the rats were left on a heating pad until recovered from anesthesia before returning to their home cage. There was no prolonged loss of consciousness beyond the normal duration of anesthesia recovery. For summary, please refer to **Figure 1** and **Table 1**.

Neuromotor Function Tests

Following 2 or 12 weeks of repeated sub-concussion impacts, neuromotor function was assessed within 48 h of sub-concussion procedure completion by utilizing established beamwalk and rotarod tests as previously described with some minor modifications (9–11). Briefly, for beamwalk, a beam of 1, 2, or 3 cm width and 30 cm length, raised 30 cm above the floor was used. The rats were placed at one end of the beam facing toward the other end, where their home cage was located. Each rat was given 3 trials on each 3, 2, and 1 cm width beam. A mean number of foot slips and latency to reach the home cage (by two front paws reaching the home cage wall) were recorded.

TABLE 1 | Summary of sub-concussion protocol.

	Procedure duration	Age	Number of impacts per session per day	Number of procedure days per week	Total procedure days	Total impacts received
Rat procedure	2 weeks	6–8 weeks	10 impacts	3 days	6 days	60 impacts
(Human equivalent*)	(0.9 years)	(2.8–3.8 years)	(N/A)	(40.5 days)	(81 days)	(N/A)
Rat procedure	12 weeks	6–18 weeks	10 impacts	3 days	36 days	360 impacts
(Human equivalent*)	(5.7 years)	(2.8–8.5 years)	(N/A)	(40.5 days)	(972 days)	(N/A)

Based on 1 rat day = 27 human days (8).

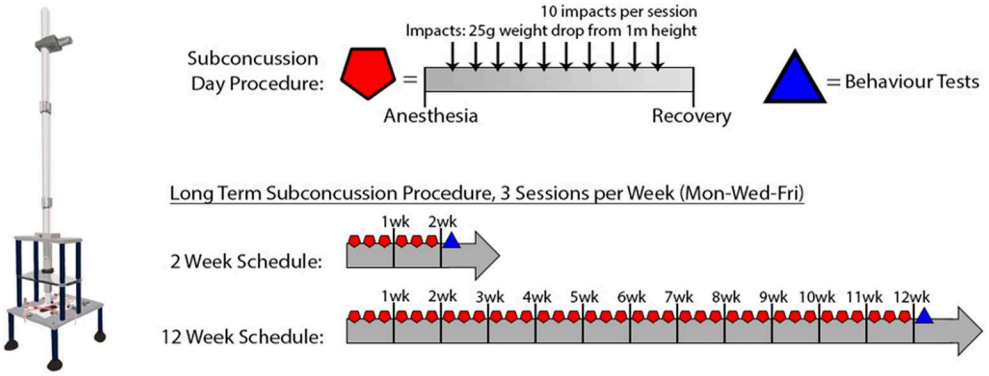

FIGURE 1 | Summary of Sub-concussion Protocol.

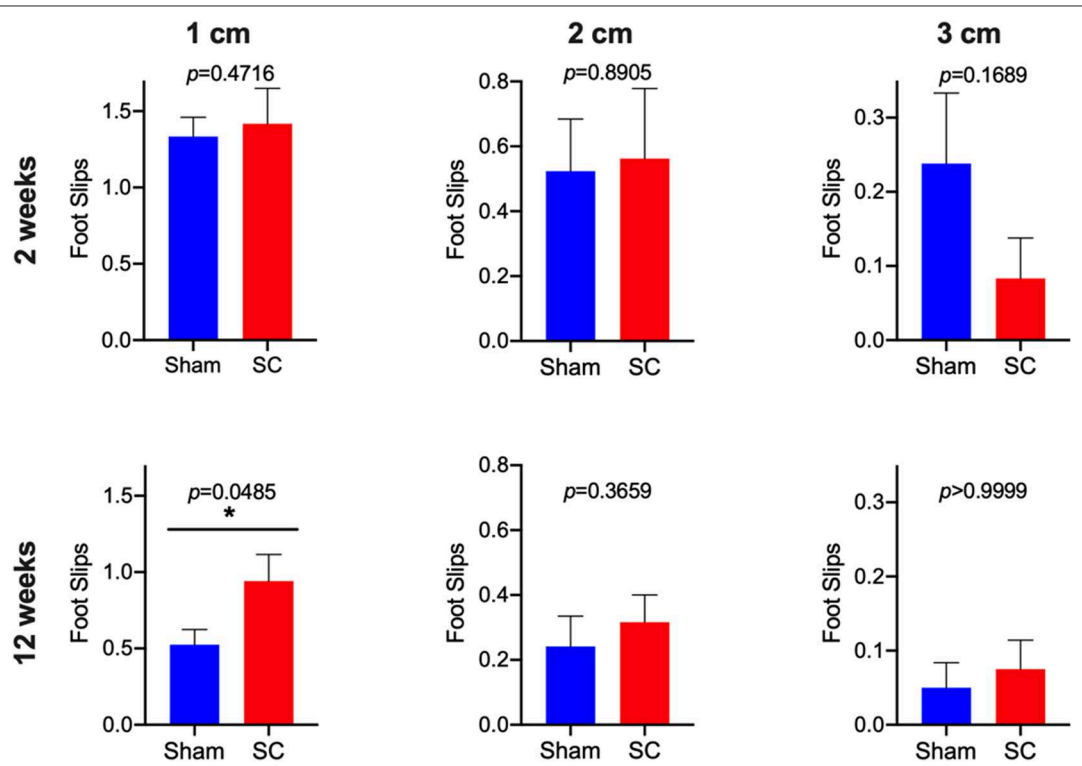

FIGURE 2 | Beamwalk Test. Neuromotor function was tested by using beamwalk test with 1, 2, and 3 cm beams in Sham and Sub-concussion (SC) group rats after 2 or 12 weeks of repeated sub-concussions. The number of foot slips are presented as a mean ± SEM of 3 trials. Statistically significant differences between Sham and SC groups at each time point was tested with unpaired *t*-test or non-parametric Mann-Whitney test (*$p < 0.05$).

The rats were acclimatized on a Rotarod apparatus (Orchid Scientific, India), rotating at 4 rpm for 60 s. At least a day of acclimatization was done with three trials per day until the rats stay on the rod without falling for 60 s. On the test day, the rats were placed on the rotating rod, accelerating from 4 to 40 rpm within 300 s. The rotation speed and latency time until the rats fell from the rod was recorded and the mean of three trials was used.

Statistical Analyses

All data are expressed as mean with standard error of the mean. Data normality was assessed with D'Agostino & Pearson test on Prism 8 (GraphPad Software, CA, US). In order to compare the neuromotor function between Sham and SC rats at each time point, unpaired *t*-tests were used for normally distributed data whilst non-parametric Mann-Whitney tests were used for the data that were not normally distributed. Statistical significance was considered to be $p < 0.05$.

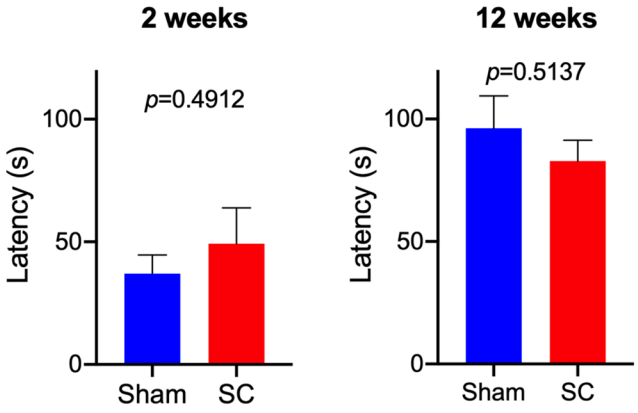

FIGURE 3 | Rotarod Test. Rotarod test was used to compare the neuromotor performance between Sham and Sub-concussion (SC) group rats following 2 or 12 weeks of repeated sub-concussions. The mean latency that the rats were able to stay on the accelerating rod was expressed as a mean ± SEM of 3 trials. Statistical significance was assessed with unpaired *t*-test or non-parametric Mann-Whitney test.

RESULTS

After receiving repeated sub-concussive head impacts for 2 weeks, the number of beamwalk foot slips whilst crossing the 3, 2, or 1 cm wide beam was comparable with the rats that received Sham procedure for 2 weeks (**Figure 2**). The latency that SC rats stayed on the accelerating rotarod was also not significantly different from the Sham group after 2 weeks of repeated sub-concussion impacts (**Figure 3**).

When the rats were given sub-concussion impacts repeatedly for 12 weeks, the SC rats showed significantly greater number of foot slips on the 1 cm width beam, compared with the Sham group rats (**Figure 2**). Whereas, a comparable number of beamwalk foot slips were observed between SC and Sham rats on the 3 and 2 cm wide beams. The rotarod test latency between the SC and Sham rats was not significantly different (**Figure 3**).

DISCUSSION

The present study is the first to test the effects of sub-concussive impacts that are repeated multiple times over an extended period, on neuromotor function in wild-type PVG rats. After 2 weeks of repeated sub-concussive head impacts, the rats showed no significant deterioration in neuromotor function, assessed by beamwalk and rotarod tests, although SC rats on 3 cm beam showed a non-significant trend of decreased foot slips. However, when the repeated sub-concussion was given for 12 weeks, the rats demonstrated significant neuromotor dysfunction when compared with the Sham group rats. This neuromotor deficit was only detectable with the narrow 1 cm beamwalk, whilst the results from wider 2 cm and 3 cm beamwalks and rotarod were comparable to the age-matched Sham rats. In beamwalk tests, the narrower beams are considered to be more sensitive in detecting motor deficits, in comparison to the wider beams (12). Moreover, beamwalk tests are reported to offer higher sensitivity over rotarod tests (13). These data collectively indicate that only after 12 weeks of repeated sub-concussive impacts, rats begin to show moderate but significant motor dysfunction, and further suggests that extended sub-concussion periods >12 weeks may induce further deterioration of neuromotor performance. The latter is consistent with clinical studies where neurobehavioral performance may only be affected by repeated sub-concussion over a prolonged term. McAllister et al. reported that collegiate contact sports players in football and ice hockey performed significantly poorer in neuropsychological and neurocognitive tests than control group athletes participating in non-contact sports (14). It is worthwhile noting that the athletes who participated in this study had no documented sports concussion during the period of study. On the other hand, Miller et al. showed that in collegiate football players, no differences in computerized neuropsychological test performance were observed between pre-season, mid-season, and post-season assessments (15), indicating that it is likely that more than one season period is required for the neurocognitive decline to become evident.

It was also noted that in the current study, that after 12 weeks of sham procedures, rats showed generally improved neuromotor function in beamwalk and rotarod tests, compared with 2 weeks Sham rats. This may result from the age of the rats when the neuromotor assessments were done, irrespective of the duration of Sham or SC procedure. However, these data were beyond the scope of the current study and may be considered further in future.

In conclusion, the current study was the first to demonstrate that in rats, frequent repeated sub-concussive impacts over an extended period, greater than 2 weeks and detectable at 12 weeks, induce modest neuromotor dysfunction. The findings provide evidence that 25 g weight drop in PVG rats provide a novel sub-concussion experimental model that may represent clinical long-term cumulative sub-concussion impacts. The advantage of this novel model is that it potentially represents clinical findings where neurostructural and neuromotor dysfunction are demonstrated following repeated sub-concussive events (e.g., soccer player's headings). A great majority of previous studies with animal models of traumatic brain injuries adopt more severe types of injuries, which are not appropriate to represent "sub-concussion." Thus, the model may be utilized to investigate relevant mechanisms and the establishment of therapeutic strategies for sub-concussion-associated neuromotor deficits in sports athletes.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Curtin Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

This study was designed and managed by AL, SR, VL, MH, MF, and RT. The animal maintenance, procedure, and sample/data collection were done by AL, SR, AW, TM, BH-M, MN, VL, MH, MF, and RT. The data interpretation and manuscript preparation were done by AL, SR, VL, MH, MF, and RT.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neurology

ORIGINAL RESEARCH
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Interleukin-1β Drives Cellular Senescence of Rat Astrocytes Induced by Oligomerized Amyloid β Peptide and Oxidative Stress

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Background: Alzheimer's disease (AD) is the leading cause of dementia. With no reliable treatment that delays or reverses the progress of AD, effective medical drugs, and interventions for AD treatment are in urgent need. Clinical success for patients thus relies on gaining a clearer understanding of AD pathogenesis to feed the development of novel and potent therapy strategies. It is well-established that inflammatory processes are involved in the pathology of AD, and recent studies implicated senescence of glial cells as an important player in the progression of AD.

Methods: We did a preliminary screen in rat astrocytes for the five most abundant inflammatory factors in neuroinflammation, namely IL-1β, IL-6, IL-8, TGF-β1, and TNF-α, and found that IL-1β could efficiently induce cellular senescence. After that, SA-β-gal staining, immunofluorescence, ELISA, qRT-PCR, and immunoblotting were used to explore the underlying mechanism through which IL-1β mediates cellular senescence of rat astrocytes.

Results: IL-1β-induced cellular senescence of rat astrocytes was accompanied by increased total and phosphorylated tau. Further experiments showed that both oligomerized amyloid β (Aβ) and H_2O_2 treatment can induce cellular senescence in rat astrocytes and increase the production and secretion of IL-1β from these cells. Subsequent mechanistic study revealed that activation of NLRP3 mediates Aβ and H_2O_2-induced maturation and secretion of IL-1β.

Conclusion: Our results suggest that IL-1β mediates senescence in rat astrocytes induced by several common adverse stimuli in AD, implicating IL-1β and NLRP3 as valuable diagnostic biomarkers and therapeutic targets for AD.

Keywords: Alzheimer's disease, neuroinflammation, interleukin-1β, senescence, astrocyte, tau, amyloid β

INTRODUCTION

There are about 50 million people in the world living with Alzheimer's or other forms of dementia (1). As the leading cause of dementia, Alzheimer's disease (AD) is a progressive neurodegenerative disorder predominantly affecting people 65 years and older. The pathological features of AD include deposition of amyloid β peptide (Aβ), neurofibrillary tangles, and neuronal degeneration. Currently, for AD treatment, there are only a few medicines (most of them are cholinesterase inhibitors) for symptomatic treatment. But so far, there is no available medical treatment that delays or reverses the clinical courses of the disease, with novel and effective therapy strategies for AD urgently needed.

The vast majority of AD cases are sporadic, with unclear etiology. Nevertheless, it is widely accepted that the occurrence and development of sporadic AD is associated with various forms of brain insults over the years. Unfortunately, the sources of adverse stimuli can be diverse and extensive, including increased oxidative stress, protein misfolding, disturbances in calcium homeostasis, and energy deficiency, etc. (2). Recently, Ehsan et al. have showed that only one night of sleep deprivation results in a significant increase in Aβ burden in the right hippocampus and thalamus in human brains (3).

It is also well-accepted that inflammatory processes are involved in the pathology of AD (4). Elevated levels of inflammatory cytokines including interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β1 are found in the brains of AD patients and animal models (5, 6). Most studies support that inflammation can promote the occurrence and development of AD, and inhibiting inflammation might help to prevent or alleviate AD (7, 8).

Cellular senescence is a state of cell growth arrest, often induced by various cellular stresses including oncogene activation, DNA damage, and telomere attrition (9, 10). Senescent cells are characterized by a flattened and enlarged morphology, increased senescence-associated β-galactosidase (SA-β-Gal) activity, and activated p16/pRB or p53/p21 pathways (11–14). In addition, senescent cells typically show a senescence-associated secretory phenotype (SASP) and secrete various pro-inflammatory cytokines (15, 16). We and the others have revealed that SASP factors can trigger senescence in surrounding cells and amplify senescence phenotypes (17–19). Interestingly, there is a significant overlap of secreted cytokines observed in AD and SASP (20).

Cellular senescence is associated with aging and is thus implicated as a potential cause of age-related neurodegenerative diseases (21, 22). Recently, Bussian et al. (23) implicated senescent cells in the etiology of AD in a P301S tauopathy mouse model, whereby senescent glial cells seemed to play a role in the initiation and progression of tau-mediated neurodegenerative diseases. About the same time, Musi et al. (24) reported a strong correlation between the presence of neurofibrillary tangles and cellular senescence in the brains of FTD associated P301L tauopathy mouse models. These papers suggest that cellular senescence plays an important role in the progression of tau-mediated neurodegenerative diseases.

As the most abundant cell type in the brain (25, 26), astrocytes control homeostasis and provide neuroprotection for the CNS (27, 28). Under physiological conditions, astrocytes supply neurons with energy, support synapses, regulate neurotransmitter levels, and release neurotrophic factors. In addition, the recent studies have shown that the astrocyte networks are essential for complex cerebral functions, such as sensation, cognition, and behavior (29, 30). According to the classical theories, astrocytes mainly respond to brain insults through a process called astrogliosis (31, 32), which usually protects but sometimes impairs the functions of the neural system when there is serious damage. Dysfunction in astrocytes was associated with the occurrence and development of AD (33, 34). Therefore, senescence of astrocytes might accelerate the AD process due to the loss of important cell functions in neurotrophic support and Aβ degradation (35–38).

Astrocytes can enter a senescent-like state *in vitro* after treatment with various stimuli (39, 40). Given that inflammatory factors can induce cellular senescence in several cell types (41, 42); the question arises as to whether inflammatory factors in the brain could induce senescence of astrocytes? To address this question, we did a preliminary screen in rat astrocytes for the five most abundant inflammatory factors in neuroinflammation, namely IL-1β, IL-6, IL-8, TGF-β1, and TNF-α, and found that IL-1β efficiently induced cellular senescence. We then further explored the roles of IL-1β in inducing astrocyte senescence and investigated the related mechanism.

MATERIALS AND METHODS

Antibodies and Other Reagents

The antibodies used in the current study are listed in **Supplementary Table 1**. The other reagents used are as follows: recombinant rat IL-1β, IL-6, IL-8, and TNF-α (PeproTech, Rocky Hill, NJ, USA); recombinant rat transforming growth factor beta-1 (MyBioSource, San Diego, CA, USA); hydrogen peroxide solution, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and DAPI (4′, 6-diamidine-2′-phenylindole dihydrochloride) (Sigma-Aldrich, St. Louis, MO, USA); Trizol and Aβ (1–42) (Camarillo, CA, USA); HiScript II One Step qRT-PCR Probe Kit (Vazyme Biotechnology, Nanjing, China); and an interleukin-1β enzyme-linked immunosorbent assay kit (Beyotime Biotechnology, Shanghai, China). Aβ oligomer was prepared using the method described in the previous study (43).

Primary Culture of Rat Astrocytes

All procedures involving rats were approved by the Jiangsu University Institutional Animal Care and Use Committee. P2-P3 neonatal SD rats were decapitated and the heads were placed into 70% alcohol for 5 min. Then, the nervous tissue, meningeal layer, brainstem, and cerebellum were removed, and the forebrains were trypsinized for 5 min at 37°C, centrifuged at 300 g for 5 min, and then cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/100 μg/ml streptomycin (Life Technologies, Grand Island, NY, USA). Cells were incubated at 37°C in an atmosphere of 5% CO_2 and 5% O_2. After 5 days, purified astrocytes were obtained through the

purification step (44). Briefly, the mixed cells were shaken at 200 rpm overnight at 37°C. After the supernatant was removed, the remaining cells were cultured with fresh medium.

SA-β-Gal Staining

SA-β-Gal staining was performed as previously described (45–47). Briefly, treated cells were fixed in 2% formaldehyde/0.2% glutaraldehyde in PBS for 5 min at room temperature. After that the slides were incubated in the staining solution (40 mM Na_2HPO_4, 150 mM NaCl, 2 mM $MgCl_2$, 5 mM K_3Fe (CN) $_6$, 5 mM $K_4Fe(CN)_6$, 1 mg/mL X-gal, pH 6.0) for 10 h at 37°C. Images were captured using an upright microscope (Nikon Eclipse, Tokyo, Japan).

Senescence-Associated Heterochromatic Foci (SAHF) Staining

The formation of SAHF is one of the most important cellular senescence markers. The method of SAHF staining was adapted from a previous study (48). Cells were fixed with 4% formaldehyde in PBS for 10 min and permeablized with 0.2% Triton X-100 for 5 min at room temperature. After that the slides were incubated with 1 μg/μL of DAPI for 5 min at room temperature and sealed. Images were obtained using the fluorescence microscope (Nikon Eclipse). Positive cells (>5 foci per cell) were counted in five different fields of each slide.

Immunofluorescence (IF) Staining

IF staining was performed as described previously (49, 50). In brief, cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were permeablized with 0.2% of Triton X-100 for 5 min and blocked with 3% BSA in PBS for 30 min at room temperature. After that cells were incubated with a selected primary antibody with an appropriate dilution for 2 h at room temperature, and then incubated with an appropriate fluorescent secondary antibody for 1 h at room temperature. Finally, the slides were stained with 1 μg/μL of DAPI for 5 min and sealed with neutral balsam. The antibodies used are listed in **Supplementary Table 1**. Images were captured using Nikon Eclipse.

Treatment of Cells

We plated 3×10^4 cells into the wells of 24-well plates with coverslips, and 3×10^5 cells into 6-cm dishes. Cells were treated with medium containing various concentrations of inflammatory factors, H_2O_2, or Aβ for 2 days. The treated cells on coverslips were used for SA-β-gal, SAHF, and IF staining. The treated cells in 6-cm dishes were used for ELISA, qRT-PCR, and Immunoblotting.

Tomato Lectin Staining

Tomato lectin staining was used to identify microglial cells (51, 52). Cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature, permeabilized with 0.2% of Triton X-100 for 5 min, and blocked with 3% bovine serum albumin (BSA) in PBS for 30 min at room temperature. Then, cells were incubated with Tomato lectin-FITC (1:500) for 2 h at room temperature and stained for 5 min with 1 μg/μL DAPI. Images were captured as above.

Enzyme-Linked Immunosorbent Assay (ELISA)

The supernatant of cells treated with or without Aβ (300 ng/mL) or H_2O_2 (30 μM) for 2 days were collected and used to quantify the concentration of IL-1β by ELISA, according to the manufacturer's instructions.

qRT-PCR and Immunoblotting

qRT-PCR was carried out using the HiScript II One Step qRT-PCR Probe Kit, according to the manufacturer's instructions. The primer sequences used in this study are listed in **Supplementary Table 2**. The endogenous control was β-actin. Immunoblotting was performed using the antibodies listed in **Supplementary Table 1**, and as described in previous studies (45, 46). β-actin was used as an internal control.

Statistical Analysis

Data are presented as mean ± SD unless otherwise noted and were analyzed for significance between groups using Student's *t*-test (two-tailed) or one-way analysis of variance (ANOVA) according to the need using GraphPad Prism version 7.00 (San Diego, CA, USA). $P < 0.05$ was considered statistically significant. $^{\#}P \geq 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

RESULTS

Primary Culture of Rat Astrocytes

After 5 days of primary culture, the mixed cells were identified using IF staining. Astrocytes, identified by their expression of GFAP (38, 53), comprised ∼58% of the primary cultured cells (**Supplementary Figure 1A**). Similarly, tomato lectin staining (54, 55) revealed a microglial cell density of ∼23%. After the purification step, the density of astrocytes increased to around 96% (**Supplementary Figure 1B**), ensuring our further research work.

IL-1β Can Induce Cellular Senescence in Rat Astrocytes

Brain inflammation often involves expression of IL-1β, IL-6, IL-8, TGF-β1, and TNF-α, thus we started our screening experiments with these commercially available inflammatory factors. Increased β-galactosidase activity and formation of SAHF in cells were used as the major markers of cellular senescence (19). The concentrations of these inflammatory factors were arbitrarily set at 1, 3, and 10 ng/ml. As shown in **Supplementary Figure 2**, while the other factors had no obvious effects on cells, IL-1β significantly increased the β-galactosidase activity and formation of SAHF in cells in a dose-dependent manner when the concentration of IL-1β was not greater than 3 ng/ml. The maximal increases in the proportions of positive cells were 2.9 and 3.2 in the SA-β-gal and SAHF assays, respectively. These results suggested that IL-1β can induce cellular senescence in rat astrocytes.

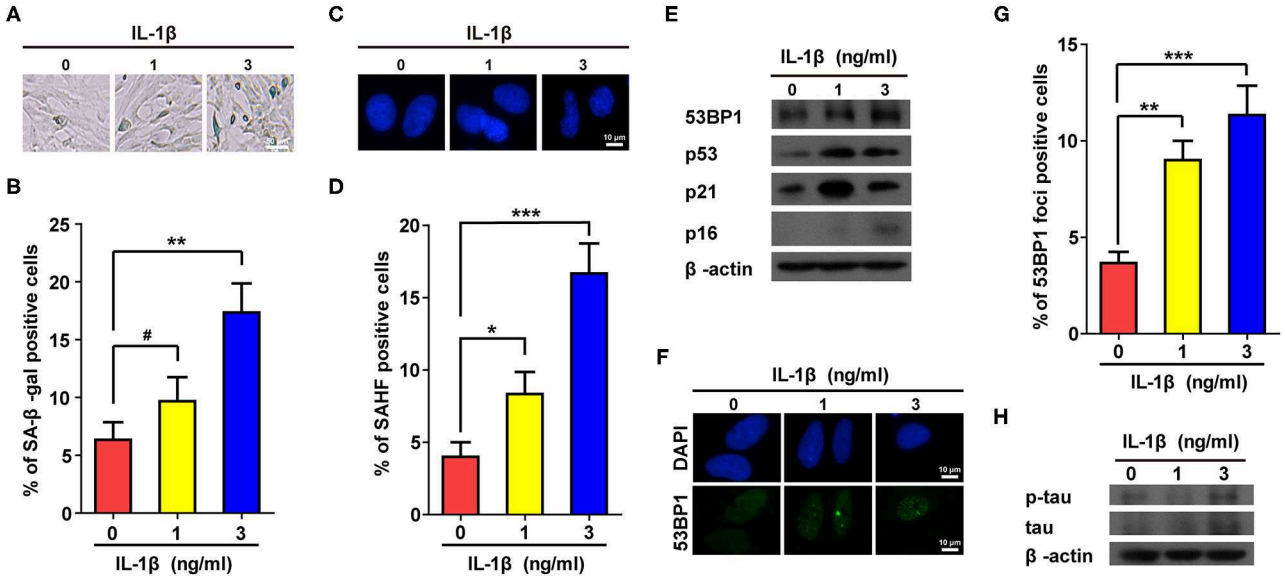

FIGURE 1 | IL-1β treatment induced senescent phenotypes in rat astrocytes. **(A,C)** Representative pictures of SA-β-gal and DAPI staining of rat astrocytes treated with different concentrations (0, 1, and 3 ng/ml) of IL-1β for 2 days. **(B,D)** Statistical analysis of **(A,C)**. **(E)** Protein levels of 53BP1, p53, p21, and p16 in rat astrocytes treated with different concentrations of IL-1β. **(F)** Representative IF staining of 53BP1 in rat astrocytes treated with different concentrations of IL-1β for 2 days, the white scale bar indicates 10 μm. **(G)** Statistical analysis of **(F)**. **(H)** protein levels of total and phosphorylated tau in rat astrocytes treated with different concentrations of IL-1β. Data indicate the mean values calculated from three independent experiments (±SD).

To confirm the above results, IL-1β (1 and 3 ng/ml) was used to treat astrocytes. Consistent with **Supplementary Figure 2**, treatment using IL-1β dramatically increased the proportions of positive cells in both SA-β-gal and SAHF assays (**Figures 1A–D**). In addition, protein markers of senescence such as p53, p21, and p16, were also up-regulated in cells treated with IL-1β (**Figure 1E**). Cellular senescence is often accompanied by accumulation of DNA damage, which is often manifested by increased numbers of foci and protein level of 53BP1. As expected, IL-1β-treated cells showed increased values for both (**Figures 1E–G**), and more importantly, the IL-1β-induced cellular senescence of astrocytes was accompanied by the increased expression and phosphorylation of tau (**Figure 1H**).

IL-1β Can Activate SASP in Rat Astrocytes

Activation of SASP pathways is an important characteristic of cellular senescence. Since IL-1β can induce cellular senescence, we speculated that this cytokine might also activate SASP pathways. As expected, IL-1β at different concentrations increased the mRNA levels of IL-6, IL-8, Matrix Metallopeptidase 3 (MMP3), and IL-1β itself to different extents (**Figure 2**).

Aβ Treatment Can Induce Cellular Senescence in Rat Astrocytes

The Aβ accumulation that is common in AD cases is considered a possible pathogenic factor. We therefore assessed the effects of Aβ treatment on rat astrocytes. As shown in **Figures 3A,B**, Aβ treatment significantly increased the portions of β-gal- and SAHF-positive cells. Although no DNA damage accumulated in these cells (**Figure 3C**), the Aβ treatment indeed induced senescence in the astrocytes that was further confirmed by increased protein levels of p21 and p16 (**Figure 3D**-a). More importantly, Aβ treatment also induced the upregulation of total and phosphorylated-tau in rat astrocytes as the cells became senescent (**Figure 3D**-b). Furthermore, the quantification of immunoblotting results showed that Aβ treatment significantly increased the protein levels of p21, p16, p-tau, and tau in a dose-dependent manner (**Figures 3E,F**).

H_2O_2 Treatment Can Induce Cellular Senescence in Rat Astrocytes

Oxidative pressure is closely related to the occurrence of AD, and H_2O_2 treatment is often used to simulate increased oxidative pressure on cells. In our study, H_2O_2 treatment induced cellular senescence manifested by increased rates of β-gal- and SAHF-positive cells (**Figures 4A,B**). Additionally, H_2O_2 treatment significantly increased numbers of 53BP1 foci (**Figure 4C**) and the protein levels of p53 and 53BP1 in cells (**Figures 4D-a, E**). These results suggested that H_2O_2 treatment caused excessive DNA damage in these cells. The elevated protein levels of p21 and p16 (**Figures 4D-b, F**) further confirmed that H_2O_2 treatment can cause cellular senescence in rat astrocytes. Interestingly, H_2O_2 treatment also upregulated total and phosphorylated-tau in these cells (**Figures 4D-c, G**).

Both Aβ and H_2O_2 Treatment Can Activate SASP Pathways and Induce Secretion of IL-1β From Rat Astrocytes

Next, we measured the mRNA levels of IL-1β, IL-6, and IL-8 in rat astrocytes treated with Aβ or H_2O_2. As expected, the mRNA levels of IL-1β, IL-6, and IL-8 all increased in cells

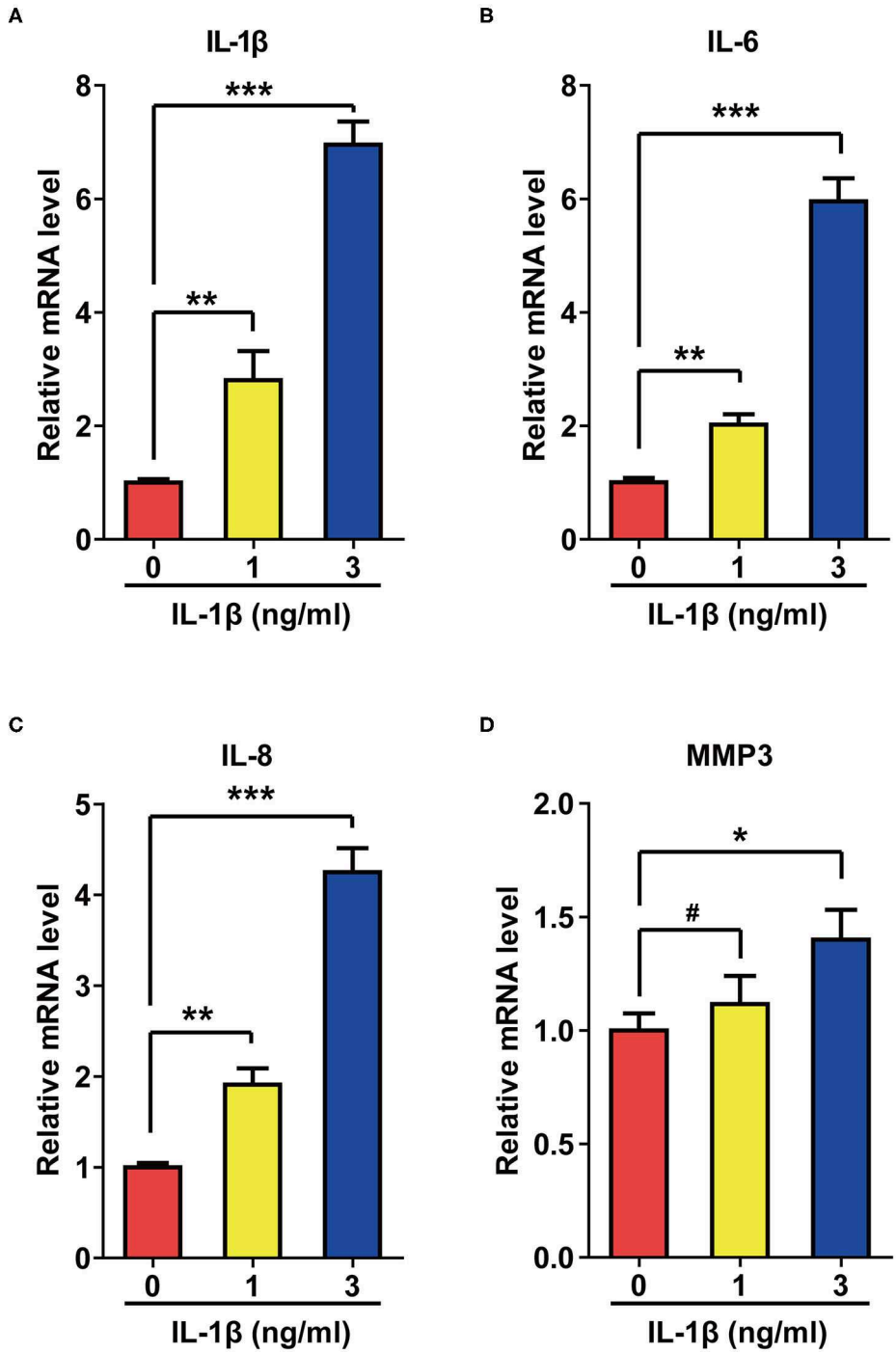

FIGURE 2 | IL-1β treatment activated the SASP pathway in rat astrocytes. **(A–D)** mRNA levels of IL-1β **(A)**, IL-6 **(B)**, IL-8 **(C)**, and MMP3 **(D)** in rat astrocytes treated with different concentrations of IL-1β for 2 days. Data indicate the mean values calculated from three independent experiments (±SD).

treated with either Aβ or H_2O_2 (**Figures 5A,B**). Consistently, immunoblotting results confirmed that both Aβ and H_2O_2 treatment increased the protein levels of mature IL-1β in cells (**Figures 5C–E**). Of note, although the protein levels of pro-IL-1β increased mildly, these changes did not reach a significant level in our experiments (**Figures 5D,E**). These results indicated that both Aβ and H_2O_2 treatment can activate SASP pathways. In addition, we found increased IL-1β protein levels in conditioned media from the astrocyte cultures (**Figure 5F**) after both the Aβ and H_2O_2 treatments. Importantly, the levels of IL-1β

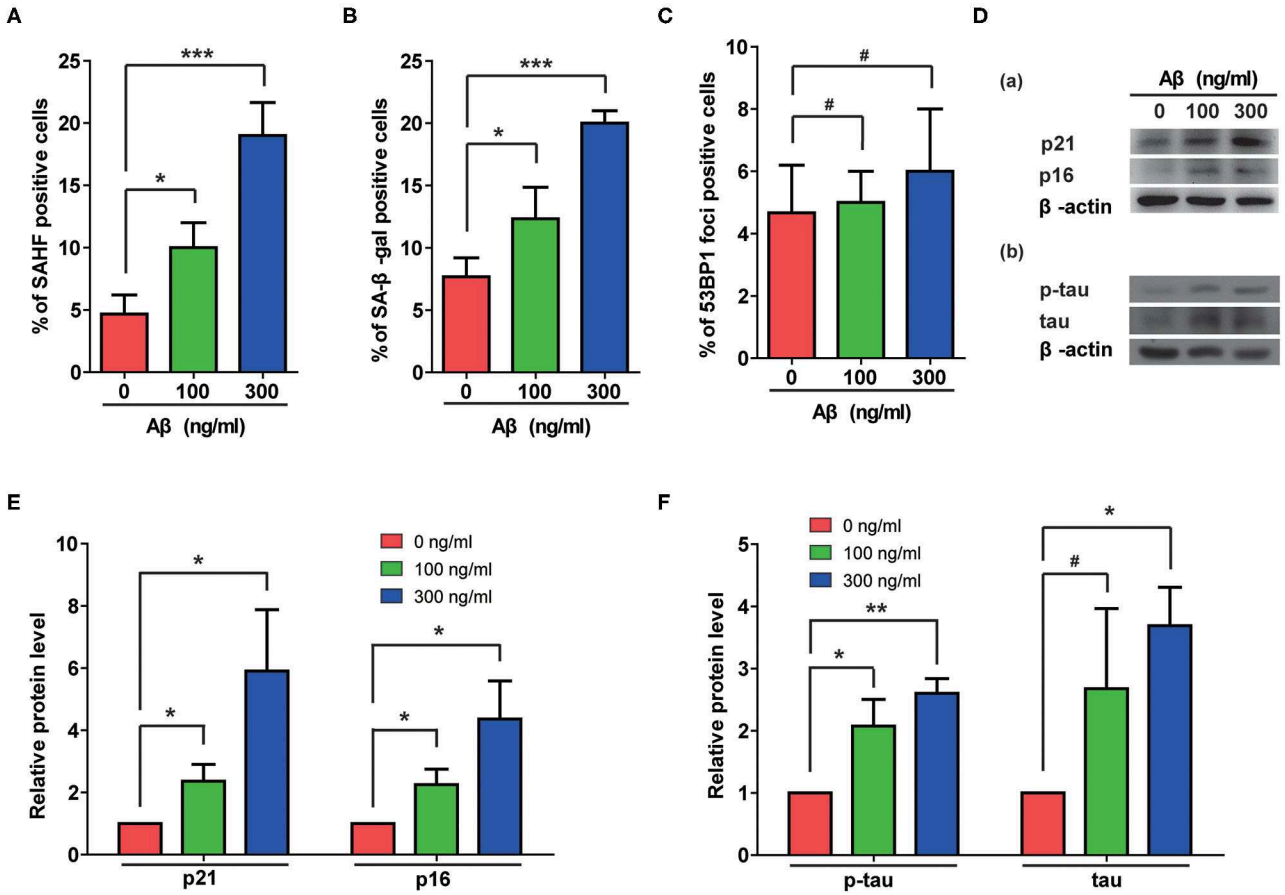

FIGURE 3 | Aβ treatment induced senescent phenotypes and upregulation of total and phosphorylated tau protein in rat astrocytes. **(A–C)** Statistical analysis of SAHF- **(A)**, SA-β-gal- **(B),** and 53BP1-foci- **(C)** positive cells in rat astrocytes treated with different concentrations of Aβ for 2 days. **(D)** Protein levels of p21 and p16 (a), p-tau and tau (b) in rat astrocytes following treatment with different concentrations of Aβ. **(E,F)** Quantification of **(D)**. Data indicate the mean values calculated from three independent experiments (±SD).

reached ∼1 ng/ml, a concentration previously shown to induce cellular senescence.

Aβ and H_2O_2 Treatment Both Can Activate NLRP3 in Rat Astrocytes

Previous studies have showed that NLR Family Pyrin Domain Containing 3 (NLRP3) senses cellular stresses, activates IL-1β transcription and promotes its maturation and secretion (56–58). We therefore accessed the expression levels of NLRP3 after Aβ or H_2O_2 treatment. As expected, both Aβ and H_2O_2 treatment significantly increased the mRNA and proteins levels of NLRP3 in our experiments (**Figures 6A–C**). Of note, compared to the control group, the increase of the protein level of NLRP3 in the group treated with 300 ng/ml of Aβ did not reach a significant level. This may be due to the large standard deviation (**Figure 6C**). Nevertheless, IF results confirmed the significant increase of NLRP3 expression in rat astrocytes treated with Aβ or H_2O_2 (**Figures 6D–F**). These data demonstrate that both Aβ and H_2O_2 treatment can increase mRNA and protein levels of NLRP3 in rat astrocytes.

DISCUSSION

It has long been speculated that cellular senescence is closely related to the occurrence and development of AD (59). Although *in vitro* evidence has been reported from time to time (60–62), the necessary *in vivo* evidence only began to accumulate recently (23, 24, 63).

In this study, we showed for the first time that the inflammatory factor IL-1β can induce cellular senescence in primary cultured rat astrocytes (**Figures 1**, **2**). Moreover, we demonstrated that both Aβ stimulation and oxidative stress can also induce senescence in rat astrocytes, and this process is accompanied by increased synthesis and secretion of IL-1β (**Figures 3–5**). Therefore, we speculate that during the development of AD, multiple adverse stimuli cause the senescence of rat astrocytes, and then IL-1β transmits and amplifies this phenomenon. Indeed, previous studies may

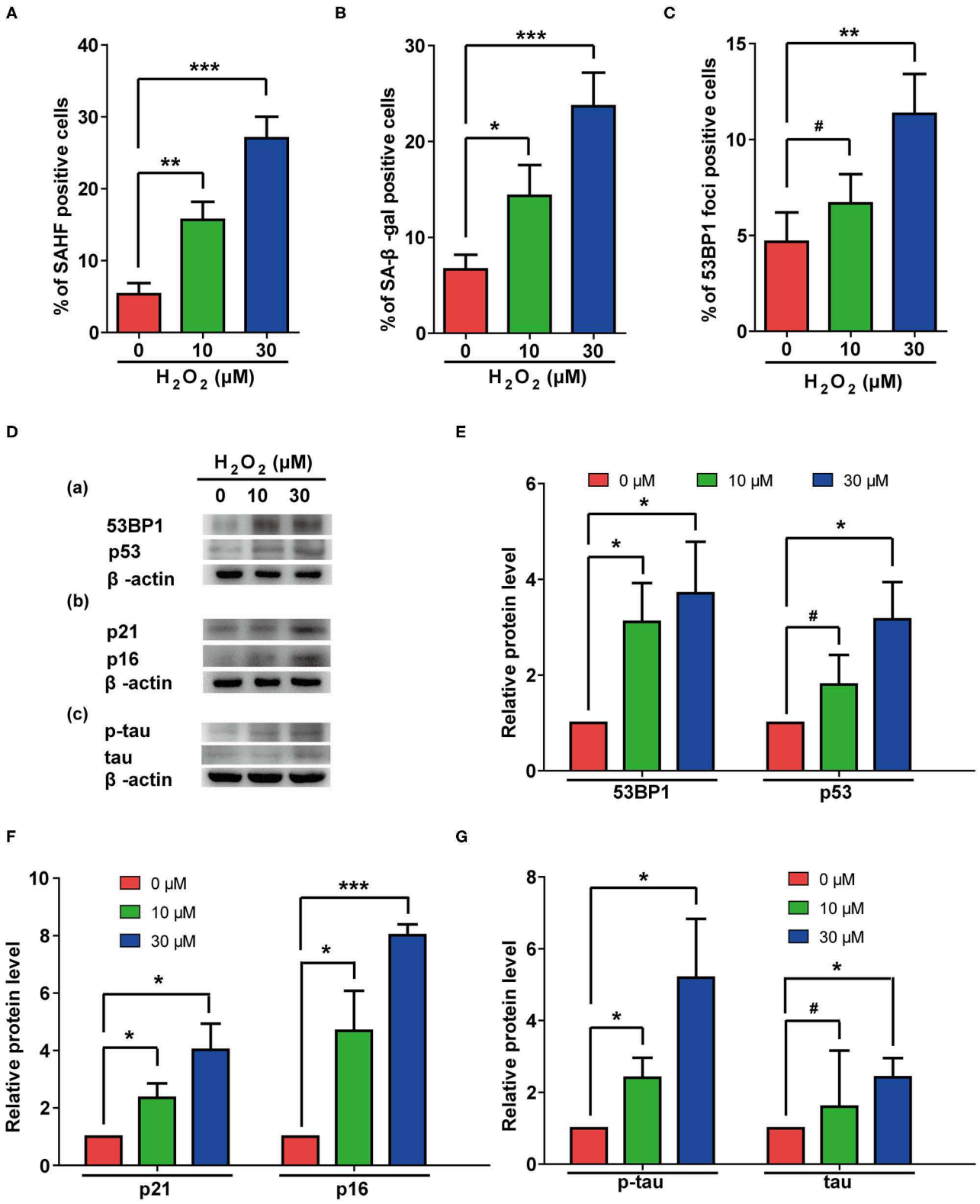

FIGURE 4 | H_2O_2 treatment induced senescent phenotypes and upregulation of total and phosphorylated tau protein in rat astrocytes. **(A–C)** Statistical analysis of SAHF- **(A)**, SA-β-gal- **(B)**, and 53BP1-foci- **(C)** positive cells in rat astrocytes treated with different concentrations of H_2O_2 for 2 days. **(D)** Protein levels of 53BP1 and p53 (a), p21 and p16 (b), p-tau and tau (c) in rat astrocytes after treated with different concentrations of H_2O_2. **(E–G)** quantification of **(D)**. Data indicate the mean values calculated from three independent experiments (±SD).

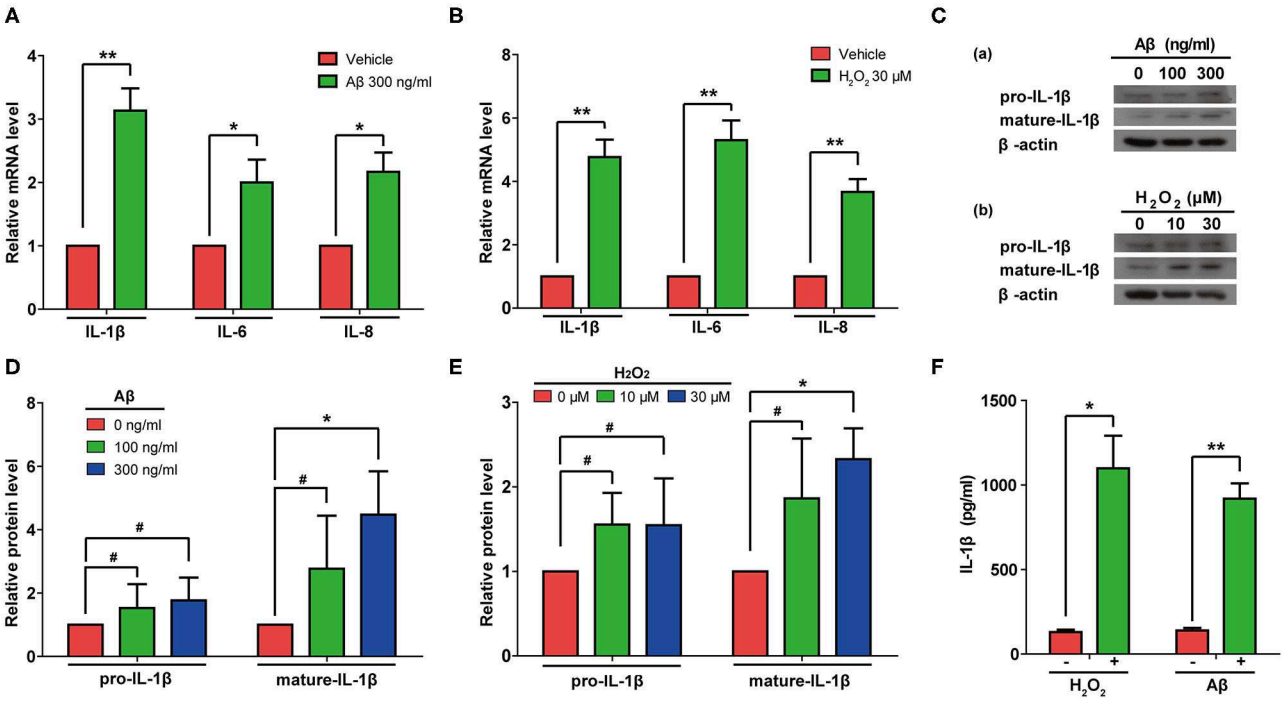

FIGURE 5 | Aβ and H_2O_2 treatment both activated the SASP pathway and induced secretion of IL-1β in rat astrocytes. **(A,B)** mRNA levels of IL-1β, IL-6, and IL-8 in rat astrocytes treated with or without Aβ **(A)** or H_2O_2 **(B)** for 2 days. **(C)** Protein levels of pro-IL-1β and mature-IL-1β in rat astrocytes treated with different concentrations of Aβ (a) or H_2O_2 (b) for 2 days. **(D,E)** Quantification of **(C)**. **(F)** ELISA quantification of the concentration of IL-1β in the supernatant of rat astrocytes treated with or without Aβ (300 ng/mL) or H_2O_2 (30 μM) for 2 days. Data indicate the mean values calculated from three independent experiments (±SD).

provide some support for this hypothesis. In one such example, Parajuli et al. (64) reported that Aβ induces IL-1β processing via the production of reactive oxygen species in microglia, however their findings could not exclude the possibility that this phenomenon is accompanied by cellular senescence in glial cells. Previous studies have shown that increased IL-1β secretion is often accompanied by activation of the NLRP3 pathway (65, 66). More specifically, Halle (67) showed that in response to Aβ, microglia secrete more IL-1β via activation of the NLRP3 pathway. Youm et al. (58) demonstrated that demonstrated that the canonical NLRP3 inflammasome links systemic low-grade inflammation to multiple age-related degenerative changes, such as thymic involution, reduced innate immune activation, and decreased brain function. Consistent with these previous findings, we also observed activation of the NLRP3 pathway in rat astrocytes treated by Aβ and H_2O_2, and NLRP3 activation (**Figure 6**), in turns, might increase the maturation of IL-1β. Our results are based on *in vitro* experiments only, *in vivo* experiments using appropriate animal models may be carried out in the future to further confirm the role of IL-1β in development of AD.

As mentioned previously, inflammation is a fundamental response to injuries in central nervous system and is associated with the occurrence and development of AD. Astrocytes were reported to secrete a broad profile of inflammatory factors (2). Consistently, our study showed that treatment with Aβ, H_2O_2, or IL-1β induced astrocytes to be senescent and to secrete several important inflammatory factors, such as IL-6 and IL-8. These results raise the following questions: In the brain, do inflammatory factors lead to cellular senescence; or does cellular senescence lead to the rise of inflammatory factors; or do these two processes crosstalk to each other as a positive feedback loop? With the existing evidence, currently we are unable to answer these questions and will pay close attention to any progress in this area.

Our recently published study (19) showed that IL-1β induces cellular senescence through EGFR activation. Interestingly, EGFR activation is common in astrogliosis (68, 69), while EGF treatment can trigger astrogliosis (70). Although it was not investigated in this study, we speculate that EGFR activation may play a role in cellular senescence of astrocytes induced by IL-1β. This raises the interesting question of whether there is some intrinsic relationship between senescence and astrogliosis in astrocytes. Due to the lack of evidence at this stage, we cannot make any judgment yet. But the question is very interesting, and worthy of further exploration.

Until now, there is no ideal animal models of sporadic AD since none of them perfectly simulates all aspects of the pathological process of AD (71). That's why many treatments and interventions had been successful in preclinical models but failed in clinical trials. Are we looking in the wrong direction? Therefore, research into novel mechanisms of AD

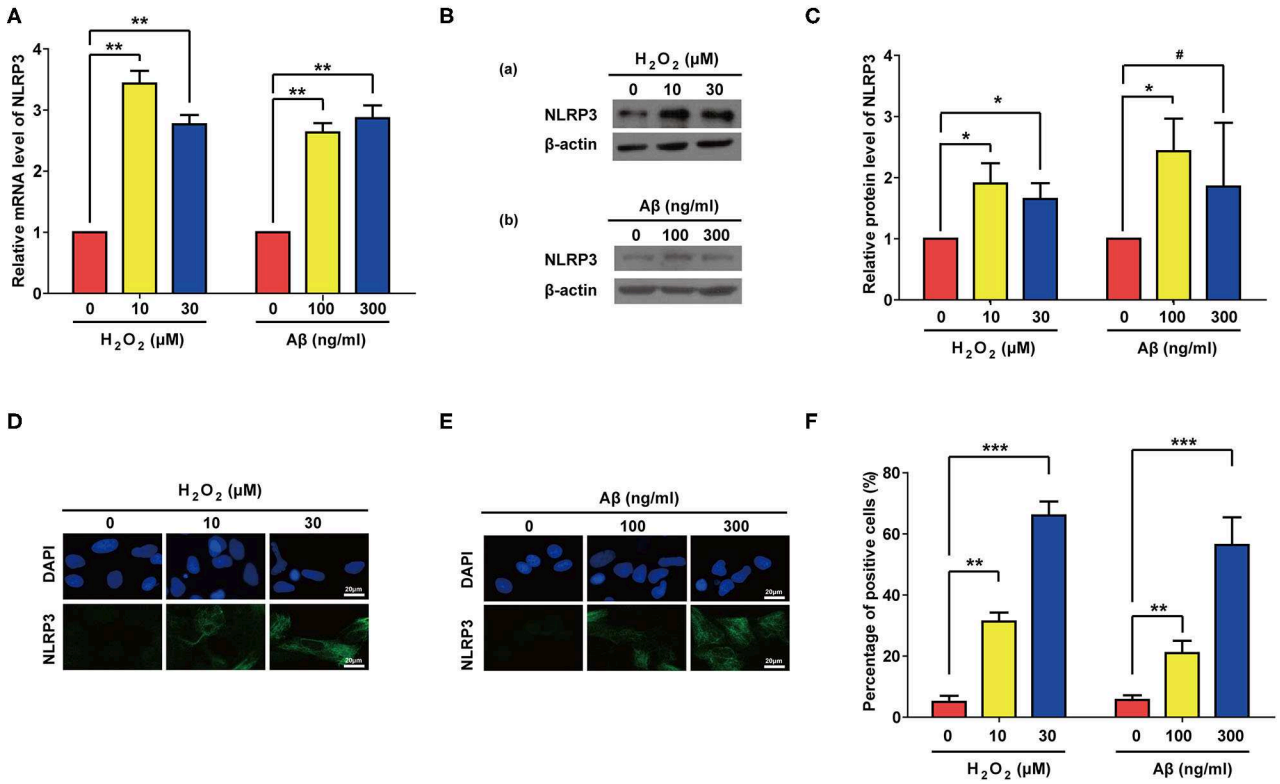

FIGURE 6 | Both Aβ and H_2O_2 treatment activated NLRP3 pathway in rat astrocytes. NLRP3 mRNA levels **(A)** and protein levels **(B)** in rat astrocytes treated with different concentrations of H_2O_2 or Aβ for 2 days. **(C)** Quantification of **(B)**. **(D,E)** Representative IF staining of NLRP3 in the treated cells, the white scale bar indicates 20 μm. **(F)** statistical analysis of **(D,E)**. Data indicate the mean values calculated from three independent experiments (±SD).

etiology is urgently needed, and astrocyte senescence induced by inflammatory factors contributing to the occurrence and progress of AD may be a very promising research direction. In addition, searching for astrocyte-specific senescence markers as early diagnostic markers for AD is an attractive research goal.

CONCLUSION

Overall, our current study, together with previous *in vivo* and clinical evidence (72–75), suggests that IL-1β and NLRP3 actively function in promoting senescence of astrocytes and could be valuable diagnosis biomarkers and therapeutic targets for AD.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal studies were reviewed and approved by the Jiangsu University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

JX, ZT, and YH conceived and designed the study. DS and WX performed experiments and acquired data. DS and YH performed data analysis. JX, ZT, and YH drafted the manuscript. JX and ZT obtained the fundings. All authors edited and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.00929/full#supplementary-material

Supplementary Figure 1 | Primary culture of rat astrocytes. **(A)** The representative pictures of immunofluorescence staining of GFAP and tomato lectin on the mixed cells. **(B)** The representative pictures of immunofluorescence staining of GFAP on purified rat astrocytes. The mean values showed in the pictures calculated from three independent experiments.

Supplementary Figure 2 | Inflammatory factor screening using SA-β-gal and SAHF assays. **(A,B)** Cells were treated with different concentrations of inflammatory factors for 2 days, and the percentages of SA-β-gal **(A)** and SAHF **(B)** positive cells were analyzed. Data indicate the mean values calculated from three independent experiments (±SD). Statistically significant differences were determined by one-way ANOVA (**$P < 0.01$, ***$P < 0.001$).

Supplementary Figure 3 | The graphic abstract.

Supplementary Table 1 | Antibodies used in this study.

Supplementary Table 2 | Primers sequences used in this study.

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CASE REPORT
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Changes in Brain Function and Structure After Self-Administered Home Photobiomodulation Treatment in a Concussion Case

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Traumatic brain injury (TBI) is a common neurological disorder among athletes. Although there are no widely accepted treatments for TBI, new investigational approaches, such as photobiomodulation (PBM), are being tested. PBM is a light therapy that uses red to near-infrared (NIR) light to stimulate, heal, and protect tissue that has been injured or is at risk of dying. Benefits following transcranial PBM treatments in animal models of acute TBI and a small number of chronic TBI patients have been reported. However, the human PBM TBI studies published to date have been based on behavioral assessments. This report describes changes in behavioral and neuroimaging measures after 8 weeks of PBM treatments. The subject was a 23-year professional hockey player with a history of concussions, presumed to have caused his symptoms of headaches, mild anxiety, and difficulty concentrating. He treated himself at home with commercially available, low-risk PBM devices that used light-emitting diodes (LEDs) to emit 810-nm light pulsing at 10 or 40 Hz delivered by an intranasal and four transcranial modules that targeted nodes of the default mode network (DMN) with a maximum power density of 100 mW/cm^2. After 8 weeks of PBM treatments, increased brain volumes, improved functional connectivity, and increased cerebral perfusion and improvements on neuropsychological test scores were observed. Although this is a single, sport-related case with a history of concussions, these positive findings encourage replication studies that could provide further validation for this non-invasive, non-pharmacological modality as a viable treatment option for TBI.

Keywords: traumatic brain injury (TBI), photobiomodulation, home treatment, cognition, neuroimaging

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INTRODUCTION

Traumatic brain injury (TBI) is a common and devastating health problem: An estimated 1.6–3.8 million concussions occur annually, and up to 10% of athletes suffer a concussion in any given sports season (1). There is presently no widely accepted treatment for TBI, although investigational approaches are being tested (2). Photobiomodulation (PBM) is a form of light-based therapy that exposes neural tissue to a low fluence of light (from <1 to >20 J/cm^2), most commonly in the red to near-infrared (NIR) wavelengths (3). Preclinical studies suggest that transcranial PBM has beneficial effects in animal models of acute TBI (4–9). Cadaver studies show that transcranial applications of NIR light can penetrate to a depth of about 40 mm in the brain (10). Thus, PBM

TABLE 1 | Timeline of the subject's concussions, PBM treatments, and testing.

Date	Event
September, 2013	1st documented concussion, sustained during a hockey game
April, 2015	2nd documented concussion, sustained during a hockey game
May, 2017	3rd documented concussion, sustained during a hockey game
November, 2017	4th documented concussion, sustained during a hockey game
May, 2018	5th documented concussion, sustained during a hockey game
February, 2019	6th documented concussion, sustained during a hockey game
March 4, 2019	Baseline MRI, HIT-6, and neuropsychological testing
March 4, 2019	Commenced transcranial/intranasal PBM treatments with Vielight Neuro Gamma
March 10, 2019	Subject advised to administer transcranial/intranasal PBM treatments with Vielight Neuro Alpha device after developing mild headaches
March 20, 2019	Subject advised to alternate using Vielight Neuro Alpha and Gamma devices after headaches resolved
April 20, 2019	Subject advised to continue transcranial/intranasal PBM treatments with just the Vielight Neuro Alpha device
May 1, 2019	Post-treatment MRI, HIT-6, and neuropsychological testing
July 1, 2020	Follow-up HIT-6 assessment

can be applied to humans non-invasively. While a few reports have described transcranial PBM treatments in chronic TBI patients (11–14), these studies were uncontrolled, the measures have been based on behavioral assessments, and the PBM treatments were administered in the laboratory or hospital. Although wider acceptance of PBM as a treatment modality for TBI is pending larger controlled studies, objective neuroimaging measures can provide data to help validate this modality as a viable treatment option for TBI. This report is the first study to use neuroimaging to investigate brain changes after 8 weeks of transcranial and intranasal PBM treatment in a subject with a history of concussion.

MATERIALS AND METHODS

The subject was a 23-year-old White non-Latino male with no family history of neuropsychiatric disorders and a favorable psychosocial background. His medical history included seasonal allergies, anisocoria, a condition where the pupils of the eyes differ in size, a fractured vertebra, a broken wrist, and six "documented" concussions. The subject was a professional hockey player, and all his injuries were hockey-related. **Table 1** summarizes the timeline of the subject's history of concussion. The subject had never been diagnosed with mild TBI (mTBI). His clinical head computed tomography (CT) and magnetic resonance imaging (MRI) exams had been negative (see **Figure 1** for representative baseline MRIs). The subject had experienced concussion-related memory gaps and feeling dazed; however, he had never lost consciousness. Thus, he was classified as a "possible TBI" by the Ohio State University TBI screen (15).

The subject approached Vielight, Inc., in Toronto, Canada about their PBM devices after his last concussion left him with a desire to "improve his mental sharpness." Prior to starting treatment, the subject's symptoms included headaches [six-item Headache Impact Test (HIT-6) (16) score = 76], mild anxiety, difficulty concentrating, and an inability to maintain attention. The subject reported previously trying acupuncture, nutritional supplements, and hyperbaric oxygen treatments for his condition. This report was undertaken as an exercise to observe potential improvements in the subject's cognitive and brain function after PBM treatments.

Vielight provided the subject with two non-thermal, non-laser light-emitting diode (LED) devices as wellness devices for his mental acuity. Vielight requested the subject to undergo neuroimaging and cognitive assessment by the first author so that observations could be made on whether objective neuroimaging data can support changes in cognition. The subject consented to this. The PBM devices used are considered non-regulated under "General Wellness: Policy for Low Risk" published by the Food and Drug Administration in September 2019.

The Vielight Neuro Alpha delivers 810-nm light pulsing at 10 Hz, 50% duty cycle; the Vielight Neuro Gamma delivers 810-nm light pulsing at 40 Hz, 50% duty cycle. Both devices have four transcranial and one intranasal LED modules designed to target nodes of the default mode network (DMN) (17), a group of strongly interconnected brain regions (18) that include the medial prefrontal/anterior cingulate cortex (targeted by the anterior transcranial LED), posterior cingulate cortex/precuneus (targeted by the central posterior transcranial LED), lateral parietal cortex (targeted by two lateral posterior transcranial LEDs), ventromedial prefrontal and entorhinal cortex, and hippocampus (targeted by the intranasal LED).

The posterior transcranial LEDs have a power of 100 milliwatts (mW) each; the anterior transcranial LED, 75 mW; and the intranasal LED, 25 mW. Each posterior transcranial LED has a power density of 100 mW/cm^2; the anterior transcranial LED, 75 mW/cm^2; and the intranasal LED, 25 mW/cm^2. The beam spot size of each LED is about 1 cm^2. The energy delivered by posterior transcranial LEDs is 60 joules (J); anterior transcranial LED, 45 J; and intranasal LED, 15 J. The energy density of the posterior transcranial LEDs is 60 J/cm^2; anterior transcranial LED, 45 J/cm^2; and intranasal LED, 15 J/cm^2. Both the PBM devices, programmed to shut off automatically after 20 min, deliver 240 J per 20-min treatment session.

Table 1 summarizes the timeline of the subject's assessments and PBM treatments. After baseline assessments, the subject began home PBM treatments every other day with the Vielight Neuro Gamma device (pulsing 810 nm at 40 Hz). This recommendation was based on a report of cognitive improvements after participants had used the Vielight Neuro Gamma device (19). However, the subject developed mild

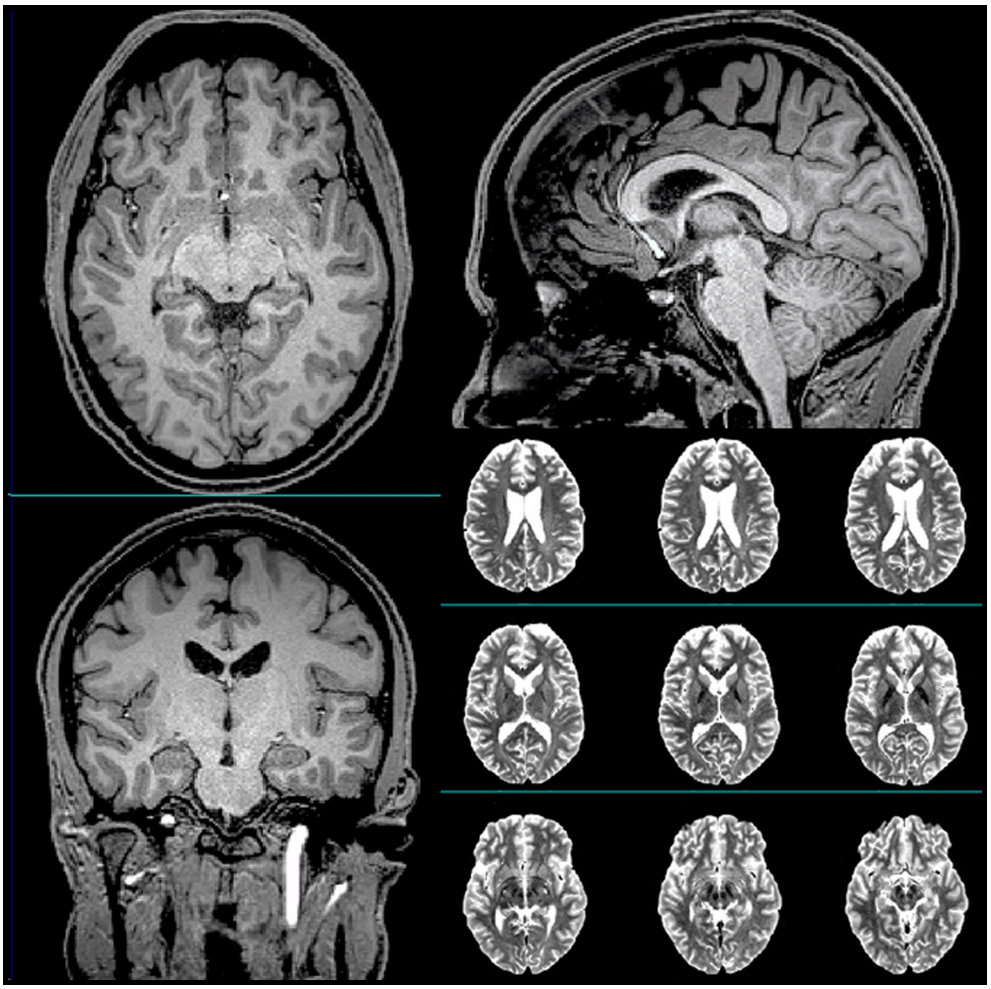

FIGURE 1 | Representative T1-weighted (axial, sagittal, and coronal) and T2-weighted (axial) anatomical scans of the subject acquired at baseline.

headaches after 1 week of using the Neuro Gamma device. Consequently, Vielight advised him to switch to the Neuro Alpha device (pulsing 810 nm at 10 Hz). When the subject's headaches resolved after 10 days, Vielight advised him to alternate between using the Neuro Alpha and Neuro Gamma devices, while keeping the every-other-day schedule of treatments. After 3 weeks, Vielight advised the subject to continue PBM treatments every other day using just the Neuro Alpha device. This decision was based on a report that PBM treatments pulsed at 10 Hz produced the most beneficial effects in an animal model of TBI (9). Although he remained in training during the PBM treatments, the subject did not play any hockey games and did not suffer any further blows to the head.

The following neuropsychological tests were administered pre- and post-treatment: California Verbal Learning Test II (CVLT-II) (20), D-KEFS Color Word Interference test (21), Trail Making Test (TMT) (22), Digit Span subtest of the Wechsler Adult Intelligence Scale-III (WAIS-III) (23), and verbal and category (semantic) fluency (24). The following scans were acquired on a Siemens 3-Tesla Trio scanner with a 32-channel receiver head coil pre- and post-treatment: structural T1-weighted 3D Magnetization Prepared Rapid Gradient Echo image [repetition time (TR)/echo/time (TE)/inversion time (TI) = 2,500/2.98/1,100 ms, $1 \times 1 \times 1$ mm^3 resolution], arterial spin-labeled (ASL) magnetic resonance images (MRI) acquired with echo-planar imaging (EPI) sequence (700 ms inversion of arterial spins, 1,900 ms total transit time of spins, 100 ms tag thickness; 13 ms echo time; field of view: 256 mm, 64×64 matrix, 24 4-mm tick axial slices; 52 tag + control image pairs with 22.5 ms time lag between slices), and resting-state functional MRI (RS-fMRI) (8-min 12-s EPI sequence with 140 time points, 3,000 ms TR, 30 ms TE, 80° flip angle, 48 3.3-mm-thick slices, $3.3 \times 3.3 \times 3.3$ mm^3 resolution; 64×64 matrix).

FreeSurfer 6.0 and FreeSurfer's longitudinal stream (25) were used to process the structural MR images and to extract volumes from anatomical regions of interest (ROIs). Total cortical gray matter (GM) volume was derived by summing the Desikan-Killiany atlas cortical ROIs bilaterally. Total subcortical GM, thalamic, and hippocampal volumes were derived from

FreeSurfer's automatic subcortical segmentation. Hippocampal subfield volumes were derived from the FreeSurfer's hippocampal subfield segmentation. The volumes from right and left hemisphere ROIs were combined.

Statistical Parametric Mapping (SPM, version 8) was used to process the ASL-MRI data, which included motion correction, aligning each ASL frame to the first frame using a rigid body transformation, and least squares fitting. Perfusion-weighted images were computed as the difference between the mean of tagged and untagged ASL data sets. To account for signal decay during acquisition and to allow for intensities in meaningful physiological units, the perfusion-weighted images were intensity scaled. After geometric distortion correction, the ASL images were aligned to structural T1-weighted images. To estimate GM perfusion and to minimize the effects of the lower perfusion in white matter on the perfusion estimates, a partial volume correction was performed using the assumption that GM perfusion is 2.5 times greater than white matter perfusion. The FreeSurfer generated anatomical ROIs were used to analyze the ASL MRI perfusion data. Mean ASL perfusion values from the cerebellum were used as a control for the meta-ROIs. Total cortical GM perfusion was derived by averaging ASL perfusion values across all the Desikan-Killiany atlas cortical ROIs bilaterally. Frontal, parietal, temporal, and occipital lobe perfusion were derived by averaging CBF values across the Desikan-Killiany ROIs that corresponded to each lobe bilaterally. Hippocampal perfusion was derived by averaging ASL perfusion values from the right and left FreeSurfer hippocampal ROIs.

CONN-fMRI Functional Connectivity toolbox version 17 (26) was used to analyze the functional connectivity data. Blood oxygen level-dependent (BOLD) signal noise from the white matter and cerebral spinal fluid was characterized with the principal component-based noise-correction "CompCor" method utilized in the CONN toolbox (27). Band-pass filtering was performed with a frequency window of 0.008–0.09 Hz and each scan was Hanning weighted (26). The ACC was chosen as a seed region to examine ROI-to-ROI functional connectivity with other brain regions. Bivariate-regression analyses were used to determine the linear association of the BOLD time series between each pair of sources in first-level analyses.

RESULTS

The subject's scores on tests of verbal learning and memory (CVLT-II), executive function (D-KEFS Color-Word Interference, TMT B, and verbal fluency), attention (digit span), and processing speed (TMT A, D-KEFS color naming and word reading) improved after 8 weeks of PBM treatments (see **Table 2**).

There were increases in the subject's total cortical GM (638.06–639.36 cm^3), subcortical GM (70.73–71.10 cm^3), and thalamic (19.11–19.19 cm^3) volumes after PBM treatment. Although total hippocampal volume decreased (9.38–9.29 cm^3) after 8 weeks, there were increases in volumes of the subiculum (968.37–996.92 mm^3), CA1 (1390.78–1395.91 mm^3), CA3 (509.17–512.72 mm^3), hippocampal–amygdala transition zone (123.17–127.26 mm^3), and fimbria (245.11–250.73 mm^3) and a decrease in the volume of the hippocampal fissure (1304.47–1299.39 mm^3).

TABLE 2 | Summary of the subject's pre- and post-treatment (Tx) neuropsychological test scores.

California verbal learning test II	**Pre-Tx**	**Post-Tx**	**Percentile Δ**
Trial 1 (*Z* score)	−1.5	0	7th→ 50th
Trial 2 (*Z* score)	−0.5	0	32nd→ 50th
Trial 3 (*Z* score)	−1	0.5	16th→ 68th
Trial 4 (*Z* score)	−0.5	0	32nd→ 50th
Trial 5 (*Z* score)	−0.5	0	32nd→ 50th
Trials 1–5 total (standard score)	43	53	25th→ 61st
Short delay free recall (*Z* score)	−0.5	−0.5	Stayed at 32nd
Short delay cued recall (*Z* score)	0	−0.5	50th→ 32nd
Long delay free recall (*Z* score)	−1	−1	Stayed at 16th
Long delay cued recall (*Z* score)	0	−0.5	50th→ 32nd
Total recall discriminability (*Z* score)	−1	0	16th→ 50th
Immediate recall discriminability (*Z* score)	−1	0.5	16th→ 68th
Delayed recall discriminability (*Z* score)	−1	0	50th→ 16th
Free recall discriminability (*Z* score)	−1	−0.5	16th→ 32nd
Cued recall discriminability (*Z* score)	−1	−0.5	16th→ 32nd
D-KEFS Color-Word Interference			
Color naming (scaled score)	11	12	63rd→ 75th
Word reading (scaled score)	10	11	50th→ 63rd
Inhibition (scaled score)	14	16	91st→ 98th
Inhibition/Switching (scaled score)	9	11	37th→ 63rd
Trail-making test			
Part A (scaled score)	13	15	84th→ 95th
Part B (scaled score)	13	14	84th→ 91st
Verbal fluency			
FAS (raw score)	59	62	84th→ 95th
Animals (raw score)	16	18	10th→ 25th
WAIS-III digit span (scaled score)	13	17	84th→ 99th

After 8 weeks of PBM treatments, there were increases in perfusion of the subject's total cortical GM (0.53–0.64 arbitrary units), frontal lobe (0.42–0.50), temporal lobe (0.62–0.72), occipital lobe (0.71–1.15), and hippocampus (0.60–0.80).

At baseline, multiple brain regions were functionally connected to the seed in the ACC (e.g., superior frontal gyrus, supplementary motor area, middle frontal gyrus, frontal pole, precentral gyrus, central operculum, supramarginal gyrus, posterior cingulate, parietal operculum, cuneus, lateral occipital, superior temporal gyrus, and insula; see **Figure 2**). After 8 weeks of PBM treatments, only the anterior insula was functionally connected to the ACC (**Figure 2**).

After 8 weeks of PBM treatments, the subject experienced subtle improvements in his headaches (HIT-6 score decreased from 76 to 70). We were unable to obtain additional measures post-treatment because the subject moved out of the country after treatment was completed. However, when we contacted him 14 months post-treatment, he reported that he continues to use the Vielight Neuro devices, albeit with less regularity. He has not played hockey and has not sustained any further blows to the head although he continues to train. His HIT-6 score at the 14-month follow-up was 50.

DISCUSSION

This report started with Vielight's attempt to help with an athlete's request to improve his "mental acuity." The athlete's diminished mental acuity may relate to his history of concussions, for which he had previously tried nutritional supplements, acupuncture, chiropractic neurology, and hyperbaric oxygen treatment.

An estimated 1.6–3.8 million sports-related mTBIs occur in athletes annually (28–30), and cognitive dysfunction from sports-related mTBI is becoming an increasing concern (31). For example, verbal learning scores measured in the post-season were lower in college athletes who participated in contact sports compared to non-contact sport athletes (24% vs. only 3.6% with low scores) (32). Furthermore, athletes who sustained the most head impacts during the season tended to have the slowest reaction times on Immediate Post-Concussion Assessment and Cognitive Testing (ImPACT) (32). Thus, it is noteworthy that the subject's scores on tests of verbal learning and memory, executive function, attention, and processing speed improved after 8 weeks of PBM treatments.

Although different forms of the CVLT-II were administered at the two time points, we cannot discount the possibility that these improvements may, at least in part, be related to practice effect. Nor can we rule out the potential influence of a placebo effect or uneven effort. One limitation of this study is we did not formally test effort. However, certain CVLT-II measures have been used to ascertain effort (33–38). For example, CVLT long delay cued recall has been used to detect poor effort in patients with TBI (35, 39). Thus, it is notable that the subject's scores on CVLT-II short and long delay cued recall declined after 8 weeks, even though his free recall scores improved. According to the CVLT-II manual, cued recall requires the retrieval of words according to a language-based strategy. Because the subject's animal fluency scores were low at baseline (10th percentile) and improved only to the 25th percentile after treatment, it is possible that this subject has some inherent language/semantic impairment. Two other CVLT-II formulas have been used to detect effort [i.e., discriminate function (35) and logistic regression (36)], although they have been shown to misclassify individuals with lower levels of education and there were no monetary incentives for incomplete effort (40). These formulas suggest that our subject exerted incomplete effort at both time points; however, he did not have a college degree and was not involved in litigation or seeking financial compensation for alleged injuries at the time of assessments. Therefore, it is possible that his attention and concentration problems resulted in uneven effort during neuropsychological testing rather than malingering.

There have been prior reports of transcranial PBM treatments in patients with chronic TBI (11–14). Naeser et al. reported cognitive improvements in 2 chronic mTBI cases after home-based transcranial PBM treatments (11) and in 11 chronic mTBI patients who participated in an open-protocol study of transcranial PBM (12). Morries et al. reported improvements in headache, sleep disturbance, cognition, mood dysregulation, anxiety, and irritability symptoms in 10 patients with chronic TBI after transcranial treatment with a Class IV high-power NIR laser (14). Cognition appeared to improve based on return to work or improved work performance. Based on the observations of the patients, their family members, and the treating clinician, quality of life also improved in the cases (14).

Because there have been no large, controlled clinical trials of PBM for TBI, widespread acceptance of PBM as a treatment option for TBI is lacking. In this respect, neuroimaging measures may provide unbiased, objective measures of functional improvements after PBM treatment. TBI-associated cerebral atrophy is a well-documented phenomenon (41). While results in mTBI have been inconclusive, observations for moderate-to-severe TBI converge toward generalized atrophy across the entire brain, on the order of 5% per year, and focal atrophy in subcortical brain regions including the thalamus and hippocampus (41). We observed increases in the subject's total cortical GM, total subcortical GM, thalamic, and hippocampal subfield volumes. Volumetric changes in a single subject after 8 weeks should be interpreted cautiously as they are within the range of observational error. Nevertheless, it is noteworthy that animal research has shown PBM to stimulate neurogenesis and protect against cell death (4, 42–44). NIR stimulates neurite outgrowth mediated by nerve growth factor (45). In animal models of TBI, NIR (810 nm) has been shown to improve neurogenesis and synaptogenesis via increase of brain-derived neurotrophic factor (4, 8, 9, 44, 46).

Significant reductions in cerebral blood flow (CBF) and lymphatic flow, particularly in the frontal and temporal lobes, have been reported in single-photon emission computed tomography studies of chronic TBI patients relative to healthy controls (47–50). In subacute mTBI patients with no significant CT or MRI abnormalities, hypoperfusion bilaterally in the frontal lobe and in the left occipital lobe has been detected with ASL MRI, a non-invasive imaging method that uses blood water as an endogenous freely diffusible tracer to

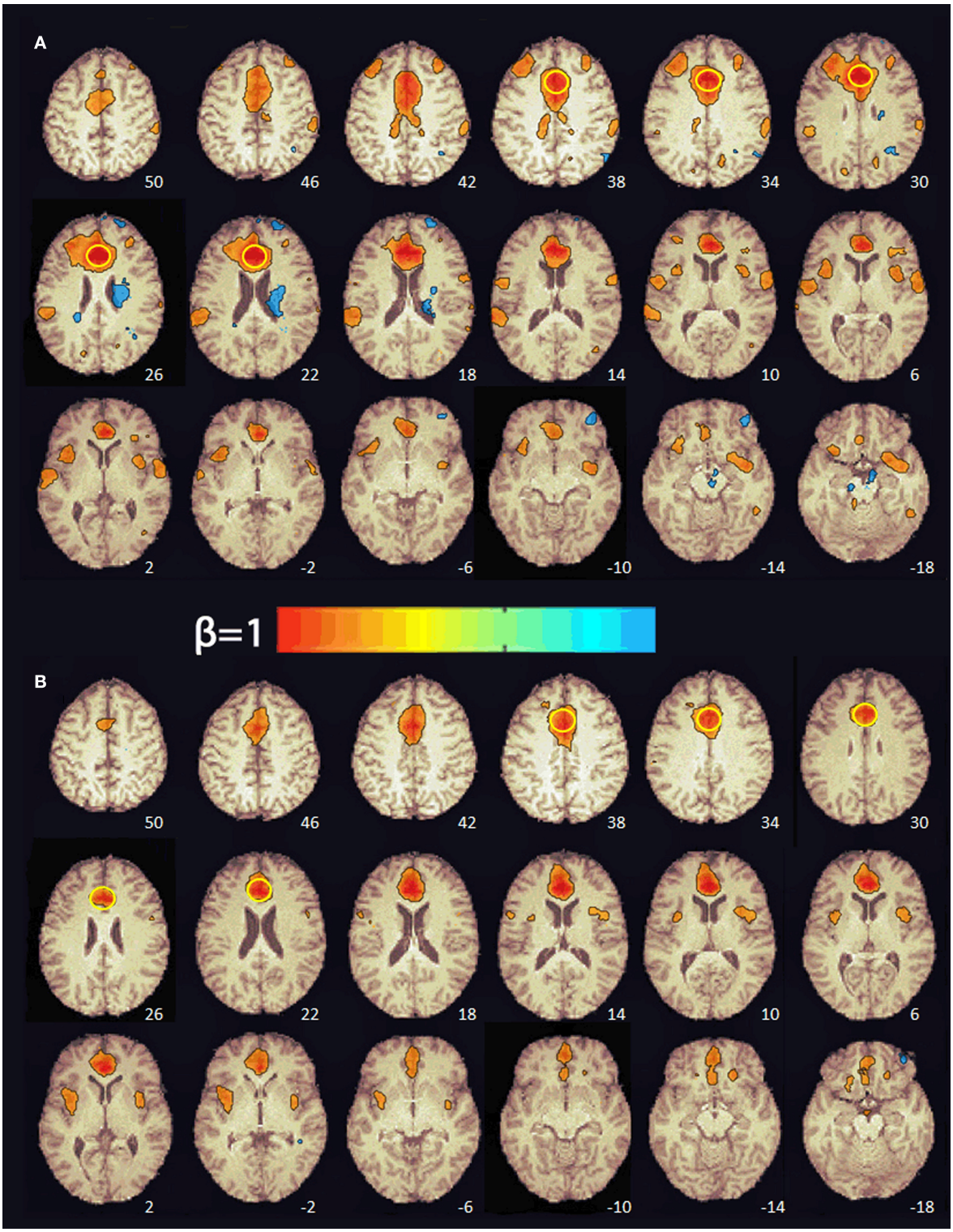

FIGURE 2 | Functional connectivity maps from the pre-treatment **(A)** and post-treatment **(B)** scans showing regions functionally connected to the seed in the anterior cingulate cortex. The numbers at the bottom right indicate the *z* coordinate (mm). The yellow circle denotes the seed in the anterior cingulate cortex. The color bar indicates the beta weight of the functional connections. The maps were thresholded at $\beta \geq 0.4$.

measure CBF (51). In the present case, there was increased perfusion in the frontal, temporal, and occipital lobes and the hippocampus after 8 weeks of PBM treatments. These findings are consistent with previous reports of PBM-related increases in local CBF (52), oxygen consumption (53), total hemoglobin, a proxy measure for regional CBF (54), regional CBF (13), and increased oxygenated/decreased deoxygenated hemoglobin concentrations (55).

RS-fMRI is the measure of spontaneous, correlated fluctuations in the BOLD signal that occur between functionally related brain regions when the brain is not engaged in a specific task (56). RS-fMRI has been used to identify intrinsic neural networks and to extract information about the connectivity and functionality of specific brain networks (57, 58). Previous RS-fMRI studies of mTBI patients have reported increases in both the number and the strength of connections between medial prefrontal regions (e.g., ACC) and other brain regions relative to healthy controls (59–66). For this reason, we selected the ACC as a seed region to investigate changes in functional connectivity pre- and post-PBM treatment. Before treatment, the subject's ACC was functionally connected to multiple brain regions in the frontal, parietal, temporal, and occipital cortex. After 8 weeks of PBM treatments, there was a decrease in both the number and the strength of the functional connections with the ACC. In fact, only the anterior insula, part of salience network (58), was functionally connected with the ACC. It has been hypothesized that the enhancements in functional connectivity seen in mTBI patients may reflect compensatory neural processes (63, 64). In a mouse model of TBI, Xuan et al. (44) found that neurological severity score, a measure of injury severity, and cognitive performance in the TBI mice improved over 4 weeks despite increases in the size of the TBI-induced brain lesions over the same period of time. They suggested that this occurred because the uninjured part of the mouse brain was steadily taking over more of the functions of the injured part of the brain. This evidence of neuroplasticity may be the compensatory mechanism that enhances functional connectivity in patients with mTBI. In the present case, the reduction in ACC functional connectivity after 8 weeks of PBM treatments may reflect a diminished need for compensatory processes after brain function normalized with PBM.

How does PBM promote brain recovery from TBI? Research has shown that PBM produces short-term increases in adenosine triphosphate (ATP) (67–70), blood (13, 54, 55), and lymphatic flow (71, 72); upregulates anti-apoptotic proteins (73–75), neurotrophins (43, 44, 76, 77), neurogenesis (44, 78), and synaptogenesis (44); and reduces edema (72, 79, 80), inflammation (81–84), and excitotoxicity (85). The best-studied mechanism of PBM centers on its effects in the mitochondria (86): In hypoxic or injured cells, the mitochondria's ability to produce ATP is reduced, likely because nitric oxide (NO), also produced in the mitochondria, can bind to cytochrome C oxidase (CCO), which inhibits respiration and displaces oxygen (87). When photons of light delivered during PBM are absorbed by CCO (88), NO is dissociated from CCO and mitochondrial inhibition is reversed (89). Photons of light delivered during PBM can also alter mitochondrial membrane permeability and ion flux, which can result in a brief increase in the production of reactive oxygen species (ROS) that shift the overall cell redox potential in the direction of greater oxidation, decreasing reactive nitrogen species (90) and increasing mitochondrial membrane potential. When this occurs, there is an increase in oxygen consumption, glucose metabolism, and ATP production (86). The brief increase in ROS can also change the activity of redox-sensitive transcription factors such as activator protein-1 and NF-κB (91), which, in turn, can activate signaling pathways and transcription factors that cause changes in protein expression (86).

This report has several limitations that should be acknowledged: First, the improvements observed on neuropsychological test scores after 8 weeks of PBM may have been, at least in part, influenced by practice and potentially a placebo effect. Second, effort was not formally tested. However, examination of certain CVLT-II measures suggests that the subject's effort on neuropsychological testing may have been uneven, possibly because of his difficulty maintaining attention and concentration. In the search for the most effective PBM regime, there were non-systematic changes in the PBM device and the combination of devices that the subject used. Because the subject's motivation for trying PBM was to improve mental acuity, Vielight initially recommended the Neuro Gamma device (pulsing NIR at 40 Hz) based on another report of the Neuro Gamma's ability to improve cognition (19). However, the subject developed mild headaches after 1 week, which resolved after he switched to the Neuro Alpha device (pulsing NIR at 10 Hz). In this regard, it is noteworthy that PBM at 10 Hz has precedent of helping to alleviate TBI symptoms (9) and pulsing PBM has been shown to modulate brain oscillations in frequency-specific ways that could influence brain functions (66). Other limitations include monitoring the subject's adherence to the PBM treatment solely through self-reports, not controlling for physical activity or diet during the treatment period, and not having measures of the variance of the imaging outcomes in healthy controls. The changes that we observed after 8 weeks in this single case may be subject to observational error. Finally, this report would have been strengthened had we tested other biomarkers that might support our hypothesis of neuroplasticity as etiology of neuroimaging changes. These limitations notwithstanding, the present case report, along with other published studies (11), suggest that it is possible for individuals with histories of concussion or TBI to self-administer PBM therapy at home. Together with previous reports of the beneficial effects of PBM in chronic TBI patients (11–14) and in preclinical studies of acute TBI (4–9), this single, sports-related concussion case suggests that larger, controlled trials of PBM for TBI and additional research on the optimal PBM treatment parameters for TBI are warranted.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

LC: conceptualization of neuropsychological and neuroimaging measures, acquisition of neuropsychological data, formal data analysis and interpretation, and writing original draft. CB: acquisition and pre-processing of neuroimaging data. LL: determined parameters of the PBM devices. CB, MK, and LL: contributed to the content of and final approval of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.00952/full#supplementary-material

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REVIEW
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The Application of rs-fMRI in Vascular Cognitive Impairment

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The incidence of vascular cognitive impairment (VCI) has been increasing for years and has become a major disabling factor in middle-aged and elderly populations. The pathogenesis of VCI is unclear, and there are no standard diagnostic criteria. Resting-state functional magnetic resonance imaging (rs-fMRI) can be used to detect spontaneous brain functional activity in a resting state, which facilitates in-depth investigation of the pathogenesis of VCI and provides an objective reference for early diagnosis, differential diagnosis, and prognostic evaluation. This article mainly reviews the principle and analysis of rs-fMRI data, as well as the progress of its application for VCI diagnosis.

Keywords: vascular cognitive impairment, resting-state functional magnetic resonance imaging, amplitude of low frequency fluctuation, regional homogeneity, functional network, default mode network, traumatic brain injury

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BACKGROUND

Vascular cognitive impairment (VCI) is a broad concept that refers to all forms of cognitive impairment (CI) associated with cerebrovascular diseases, which covers the full spectrum from vascular mild cognitive impairment (vascular MCI) to vascular dementia (VaD) and includes cases with mixed pathologies, such as mixed vascular and AD-type pathologies (1–3). According to the Vascular Impairment of Cognition Classification Consensus Study (VICCCS), VCI includes two types (4, 5): mild VCI and major VCI (VaD); major VCI includes the following four subtypes: post-stroke dementia (PSD), subcortical ischemic vascular dementia (SIVD), multi-infarct (cortical) dementia (MID), and mixed dementias (MixD). With the aging population, the incidence of dementia has increased over the years. VCI is the second most common cause of dementia, only after Alzheimer's disease (AD) (6, 7). VCI not only affects patients' quality of life but also causes a heavy burden to families and society.

Currently, the pathogenesis of VCI is not completely clear, and there is no unified diagnostic standard. The sensitivity and specificity of VCI diagnosis based on clinical manifestations and various scales are not high. The heterogeneous nature of cerebrovascular disease makes it challenging to clarify the pathological substrates of VCI. Studies indicated that the pathological substrates of VCI mainly involved arteriolosclerosis, small or lacunar infarcts, microinfarcts, perivascular space dilation, myelin loss, leptomeningeal cerebral amyloid angiopathy, and so on. In recent years, cerebral small vessel disease (SVD) has been recognized as an important substrate of CI. SVD is characterized by arteriolosclerosis, lacunar infarcts, cortical and subcortical microinfarcts, and diffuse white matter changes (including myelin loss and axonal abnormalities) (8–14). In addition, some biomarkers such as the cerebrospinal fluid (CSF) matrix metalloproteinases (MMPs), CSF/serum albumin quotient (QA), and possibly blood inflammatory cytokines and adhesion molecules, etc., can indirectly reflect the pathophysiological process of VCI (15–17).

MRI should be the first choice for patients with suspected VCI. The evaluation contents include brain atrophy, infarction, white matter hypertensities (WMHs), and hemorrhage (18). In recent years, fMRI development has provided important new research methods for studying the relationship between cerebrovascular diseases and CI. Resting-state functional magnetic resonance imaging (rs-fMRI) can be used to detect spontaneous brain functional activity in a resting state, which facilitates in-depth investigation of the pathogenesis of VCI and provides an objective reference for early diagnosis, differential diagnosis, and prognostic evaluation. This article mainly reviews the principle and analysis of rs-fMRI data, as well as the progress of its application for VCI diagnosis.

FUNDAMENTAL PRINCIPLES OF RS-FMRI

Blood-oxygen-level-dependent fMRI (BOLD-fMRI) can be used to acquire brain activity images based on hemodynamic changes in different functional brain areas (19). When the brain blood flow in the activation zone significantly increases, the blood oxygen level and relative proportion of oxygenated hemoglobin and deoxyhemoglobin will increase, resulting in changes in compliance in the corresponding region. Compliance changes can cause MRI signal variations. Brain maps with different functional states can be drawn through relevant data processing (20–23). BOLD-fMRI has mainly two modes: task-state and resting-state. Task-state fMRI requires task design for the subjects, and the subjects need to fully cooperate to accurately complete the task. rs-fMRI does not require specific task design for the subjects, and subjects only need to be awake with closed eyes, breathe calmly, minimize active and passive physical movements, and avoid any thinking activities. rs-fMRI detects spontaneous neuronal activity in the baseline state of the brain by MRI scan and determines the network connection of relevant brain regions, reflecting the spontaneous functional activities in the resting state (24). Compared with task-state fMRI, rs-fMRI has the characteristics of being relatively simple, easy to operate, easy to accept by subjects, and easy to conduct for large sample size studies. Because fMRI is non-invasive, has good temporal and spatial resolution, and can visibly reflect the features of relevant brain functional changes, it has been widely used in brain functional imaging studies.

RS-FMRI DATA ANALYSIS

rs-fMRI mainly analyzes the abnormal activities of the brain region of the subject from three aspects: local brain function, functional connectivity (FC), and functional network. For local brain activity, the amplitude of low frequency fluctuation (ALFF) and regional homogeneity (ReHo) are used to analyze spontaneous neural activity in the local brain region from the perspective of functional separation. Brain FC analysis generally uses analytical methods that target all limited numbers of brain regions, including seed-based correlation analysis for the region of interest (ROI), independent component analysis (ICA), principal component analysis (PCA), dynamic causal analysis (DCM), and Granger causal analysis, among which seed-based correlation analysis and ICA are more commonly used. A brain functional network is a collection of functional dependencies among anatomically identified brain regions. It shows many topological properties, for example, small-world network attributes, among others. Recently, graph theory-based network analyses have been widely used in brain functional network research.

ALFF

ALFF was proposed by Zang et al. (25) to reflect the intensity of spontaneous synchronized neural activity of various voxels in the low-frequency range (0.01–0.08 Hz) from the perspective of energy metabolism (25). Increased ALFF indicates an increase in excitability in the brain region. ALFF is correlated with cognitive function in the subject (26). ALFF is more sensitive to physiological noise. The fractional ALFF (fALFF), which is the ratio of the low-frequency power spectrum to the power spectrum of the entire frequency range, can significantly inhibit the physiological noise in the cistern in rs-fMRI and improve the sensitivity and specificity of spontaneous brain activity detection (27).

REHO

The ReHo method uses Kendall's coefficient concordance (KCC) to measure the consistency of time series between voxels in the brain. The ReHo map of the subject was obtained by calculating the KCC value of each voxel in the whole brain (28). ReHo reflects the functional status of the whole brain, but it cannot specifically indicate the activity status of each brain region. The increase in ReHo value means that the neuron activity tends to be synchronized in time, and vice versa, indicating that the neuron activity consistency is reduced, suggesting that neuron activity in the corresponding brain regions is abnormal.

ICA

ICA is a type of data-driven method that has been widely used to analyze FC in the brain (29, 30). This method decomposes the signal into multiple spatially independent components and identifies a FC between brain regions with a relatively large signal projection on the same component. ICA is not necessary to preselect the seed-based region of interest and can separate the independent components corresponding to each functional network, as well as the impact of noise such as head motion and respiration on the signal. ICA can determine the spatial distribution of the network, but it cannot measure the connection strength of the brain regions.

SEED-BASED CORRELATION ANALYSIS

Seed-based correlation analysis is the most basic method for early studies of FC, and it is also a common analytical method for rs-fMRI study of FC, which is a supplement to ICA. It has been

adopted in many studies (31, 32). The seed-based correlation analysis first determines the seed-based coordinates of a network, extracts the BOLD signal from the seed area, and determines the temporal correlation between the ROI and other brain voxels.

This method can reflect the strength of association between brain regions and has the characteristics of being simple, sensitive, and easy to compare differences between groups. However, the analysis results depend on the selection of seed areas and cannot simultaneously process multiple systems (33).

GRAPH THEORY-BASED NETWORK ANALYSES

The brain is a complex and highly efficient network composed of spatially distributed but functionally connected brain regions. The brain network enables the coordination between neuron activities and between different brain regions. A complex network consists of nodes (representing the structural regions of the brain) and the edges connecting the nodes (34). The brain network has efficient small-world network attributes (35–41). The small-world network has a high clustering coefficient (Cp) and a short characteristic path length (Lp), allowing the transmission and processing of functional network information to be performed efficiently (35, 36). Small-world networks reflect brain functional differentiation and information integration, as well as the adaptability of the human brain to a variety of strong stimuli (37, 38). Threshold selection directly affects the statistical characteristics and topology of brain networks (39, 40). There are some groups of nodes in the network with tight internal connections but sparse external connections, called modules. The modularity Q reflects the level of functional separation of the brain network, and the modular structure allows a more detailed differentiation of the roles and statuses of different nodes. Graph theory analysis is a high-level network analytic method that is widely used in complex brain network research.

APPLICATION OF RS-FMRI IN THE VCI STUDY

Local Brain Activity

Local brain activity abnormalities are associated with CI. In a resting state, the default mode network (DMN) is very active, and the activities of these brain regions significantly weaken under certain cognitive tasks (42). These regions include the medial prefrontal cortex (mPFC), posterior cingulate cortex (PCC), precuneus, anterior cingulate cortex (ACC), parietal cortex, and, in a minority of studies, also the hippocampus (43). Among the anatomical parcellation units, the ordering of the low-frequency oscillation (LFO) amplitudes is significant, showing a significant spatial distribution (44). DMN is closely related to the extraction of episodic memory, environmental alertness, cognition, and emotional processing. Many studies indicated that LFO amplitude measurement can describe the intergroup features of the rs-fMRI dataset and contribute to investigations of the pathogenesis of VCI (45–48). Liu et al. (45) investigated the ALFF alteration of whole brain in 30 patients with SIVD and 35 control subjects using structural MRI and rs-fMRI scan. Their study showed that the ALFF levels in the bilateral ACC, PCC, mPFC, inferior parietal lobe (IPL), occipital lobe, and adjacent precuneus were higher than the global mean ALFF levels in both groups. Compared to the controls, SIVD patients presented lower ALFF levels in the bilateral precuneus and higher ALFF levels in the bilateral ACC, left insula, and hippocampus. The ALFF values of the left insula were negatively correlated with the Montreal Cognitive Assessment (MoCA) and Mini-Mental State Examination (MMSE) scores of SIVD patients.

Yi et al. (46) investigated changes in functional amplitude of spontaneous LFO and FC density in 26 patients with subcortical vascular mild cognitive impairment (svMCI) and 28 healthy controls (HCs). They found that the svMCI-related changes were mainly located in the DMN. Compared with the HCs, the patients with svMCI presented decreased LFO amplitudes in the anterior part of the DMN and increased LFO amplitudes in the posterior part of the DMN.

ReHo values were highly correlated with cognitive function. Studies have shown that ReHo of related brain regions of VCI patient was significantly decreased (**Figure 1**). Peng et al. used ReHo analysis for post-stroke patients with poor cognitive function (PSPC) (49). Compared with the control group, PSPC patients had significantly reduced ReHo values in the bilateral anterior cingulate gyrus and left posterior cingulate gyrus/precuneus. Another study (50) explored the relationship of white matter lesions (WMLs) with CIs from the aspect of cortical functional activity in 16 patients with ischemic WMLs and 13 controls using a ReHo approach. This study showed regions with altered ReHo values in patients with ischemic WMLs to be involved in DMN, frontal–parietal control network (FPCN), dorsal attention network (DAN), motor network, and right temporal cortex. Moreover, some altered regions belonging to DMN, FPCN, and motor network were correlated with MMSE and MoCA scores significantly. It is very interesting to find that the decreased ReHo was mainly in the anterior brain regions while increased ReHo was in the posterior brain regions. This result may indicate a failure in downregulation of spontaneous activity in posterior regions. Similar results were obtained in the other studies (51, 52). The above results indicate that changes in ReHo values in related brain regions can be used as imaging markers for VCI.

Brain FC

Because DMN FC abnormalities are closely related to CI, and PCC is the core part of DMN, PCC is often selected as the ROI to study its connection with other brain regions (53–55).

Carotid artery stenosis (CAS) without transient ischemic attack (TIA) or stroke has been previously considered asymptomatic. However, many studies have shown that asymptomatic CAS (aCAS) is not truly asymptomatic, and these patients are considered cognitively impaired in functional performance, psychomotor speed, and memory testing. Wang et al. (56) performed FC analysis in 19 aCAS patients and 24 HCs. Compared with the controls, aCAS patients exhibited significantly poorer performance on global cognition, memory, and executive function, and suffered decreased connectivity to the PCC in the anterior part of DMN. Sun et al. (57) investigated the changes in the resting state of 16 patients with VCI. The

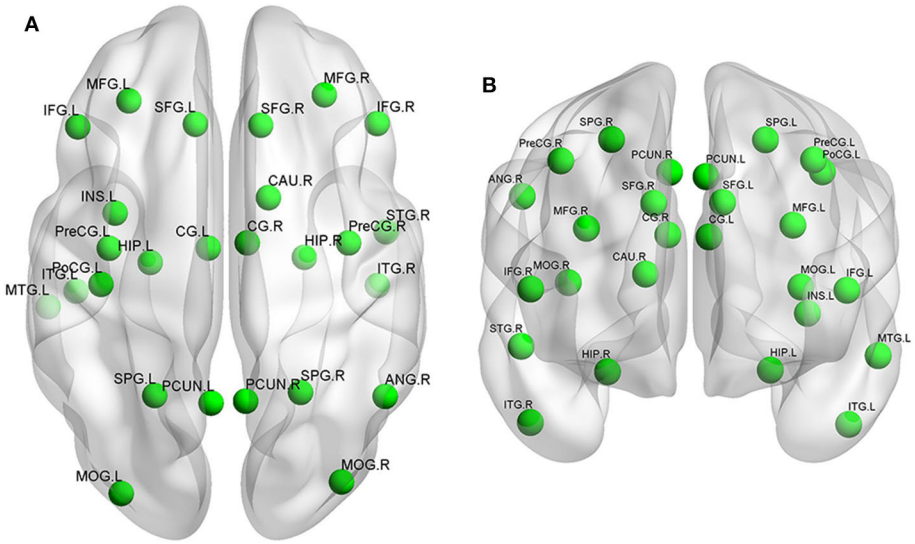

FIGURE 1 | Neural networks involved in VCI mainly include the prefrontal cortex, temporal cortex, and hippocampus. The functional changes are shown in the **(A)** superior view and **(B)** anterior view. L, left; R, right; SFG, superior frontal gyrus; MFG, middle frontal gyrus; IFG, inferior frontal gyrus; PreCG, precentral gyrus; PoCG, postcentral gyrus; SPG, superior parietal gyrus; ANG, angular gyrus; PCUN, precuneus; MOG, middle occipital gyrus; STG, superior temporal gyrus; MTG, middle temporal gyrus; ITG, inferior temporal gyrus; INS, insular cortex; CG, cingulate gyrus; CAU, caudate; HIP, hippocampus.

patients presented FC decrease in the left middle temporal gyrus, the left anterior cingulate/left middle frontal gyrus, the right caudate, the right middle frontal gyrus, and the left medial frontal gyrus/paracentral lobule. However, there were also some regions that showed increased FC, including the right inferior temporal gyrus, the left middle temporal gyrus, the left precentral gyrus, and the left superior parietal lobule.

Many studies have shown (58–61) that FC abnormalities in VCI subjects are closely related to CI (**Figure 1**). DMN is the key brain region for post-stroke CI. Yi et al. (46) investigated changes in FC density in patients with svMCI. They found that the FC of DMN continued to decline in terms of FC density. Ding et al. (59) investigated the differences of FC in the DMN in 18 stroke patients with and without post-stroke cognitive impairment (PSCI vs. Non-PSCI). They found that both PSCI and Non-PSCI patients showed significantly decreased FC in the PCC/PCu and increased FC in the mPFC as well as left hippocampus. However, Non-PSCI patients showed more significantly increased FC in the mPFC and hippocampus than PSCI patients did.

However, some studies (61, 62) have shown that VCI patients not only present an abnormal FC of the DMN but also show abnormal FC of other multiple resting state networks (RSNs). Li et al. (61) studied 21 TIA patients who suffered from an ischemic event and 21 HCs using cognitive tests, psychiatric tests, and fMRI. Their results showed that TIA patients showed both decreased and increased FC in DMN and self-referential network (SRN) and decreased FC in DAN, central-executive network (CEN), core network (CN), somato-motor network (SMN), visual network (VN), and auditory network (AN) than HCs did. This study indicated that TIA is a disease with widely abnormal brain networks. Similar results were obtained by Wang et al. (57). DMN is a collection of regions affecting cognitive recovery. Park et al. (63) for the first time examined longitudinal changes in the DMN during the 6 months after stroke. In their study, the stroke patients demonstrated obviously decreased DMN connectivity of the PCC, precuneus, medial frontal gyrus, and IPLs at 1 month after stroke, and the DMN connectivity of these brain areas was almost restored at 3 months after stroke, suggesting that the period is important for neural reorganization. The DMN connectivity of the dorsolateral prefrontal cortex in the contralateral hemisphere is significantly associated with cognitive function recovery, which may be a compensatory process to overcome the CI caused by brain injury (63). These findings will help to further understand underlying VCI mechanisms and suggest that resting-state network connectivity can serve as an imaging biomarker for VCI.

Brain Functional Networks

Studies of cerebral leukoencephalopathy, stroke, and carotid stenosis with or without VaD leading to VCI disease (64–66) have shown that the decline in cognitive function was correlated to the abnormal small-world network attributes and topological parameters of the brain. Yu et al. (67) conducted graph theory-based network analyses of 23 subcortical VCI (SVCI) patients and 20 HCs. The results showed that the brain functional networks of the SVCI patients and control groups showed small-world attributes within the threshold range ($0.15 \leq$ sparsity ≤ 0.40). The global topological organization of the functional brain networks in SVCI was significantly disrupted. The reduction of the SVCI active area occurred mainly in the frontal lobe, while the subcortical area showed an increase in characteristic Lp, potentially compensating for the inefficiency of the functional network. Yi et al. (34) obtained similar conclusions.

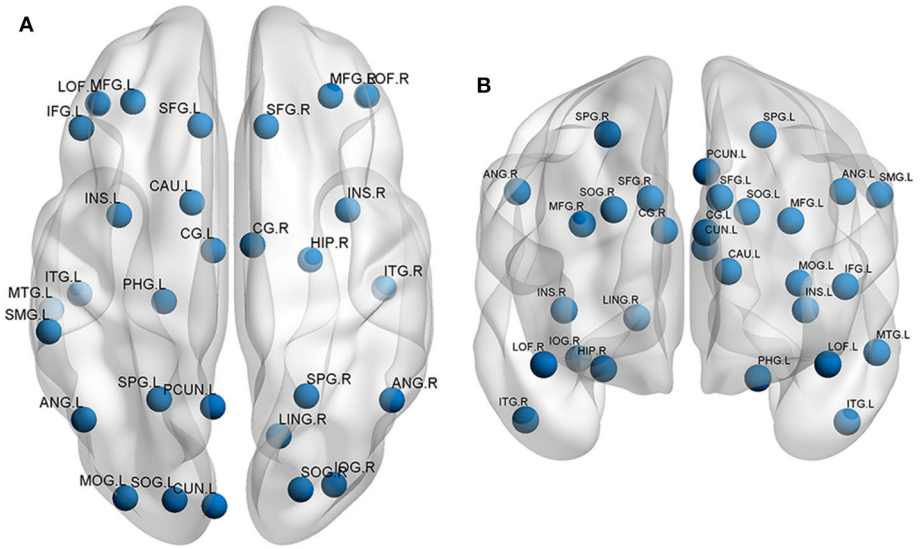

FIGURE 2 | Neural networks involved in traumatic brain injury mainly include the prefrontal cortex, temporal cortex, occipital cortex, and cingulate gyrus. The functional changes are shown in the **(A)** superior view and **(B)** anterior view. L, left; R, right; SFG, superior frontal gyrus; MFG, middle frontal gyrus; IFG, inferior frontal gyrus; LOF, lateral orbitofrontal gyrus; SPG, superior parietal gyrus; SMG, supramarginal gyrus; ANG, angular gyrus; PCUN, precuneus; SOG, superior occipital gyrus; MOG, middle occipital gyrus; IOG, inferior occipital gyrus; CUN, cuneus; MTG, middle temporal gyrus; ITG, inferior temporal gyrus; PHG, parahippocampal gyrus; LING, lingual gyrus; INS, insular cortex; CG, cingulate gyrus; CAU, caudate; HIP, hippocampus.

APPLICATION OF RS-FMRI IN MILD TRAUMATIC BRAIN INJURY (MTBI) AND OTHER COGNITIVE DISORDERS

In recent years, the incidence of TBI has increased. Although the majority of patients with mTBI had mild or no significant brain damage and the conventional imaging results were usually negative, some patients had a certain extent of CI. Neural networks involved in TBI mainly include the prefrontal cortex, temporal cortex, occipital cortex, and cingulate gyrus (68, 69) (**Figure 2**). One study (63) showed that patients with mTBI had a significant decrease in ALFF in the cingulate gyrus, middle frontal gyrus, and superior frontal gyrus; the cingulate gyrus ALFF was significantly positively correlated with the working memory index. In addition, FC of the thalamus, caudate nucleus, and right hippocampus of these patients was significantly reduced, and a significantly positive correlation between FC in the left thalamus and the left middle frontal gyrus and WMI. Zhu et al. (69) used rs-fMRI to study the longitudinal changes in function and structural connections of the DMN in mTBI patients at 24 h, 7 days, and 30 days. This study showed that the general trend of increased DMN FC occurred on the first day, which decreased significantly on day 7 and partially recovered on day 30.

In addition, studies have found that Parkinson's disease (PD), Huntington's disease, schizophrenia, major depressive disorder, type 2 diabetes mellitus (T2DM), chronic obstructive pulmonary disease (COPD), lung cancer following chemotherapy, hepatic encephalopathy, kidney deficiency syndrome, and other diseases can also cause CI (70–79), but the relationships between these diseases and cognitive function and the pathogenesis of CI are still unknown, necessitating further in-depth studies. rs-fMRI will play an important role in the exploration of these unknown areas.

CONCLUSION

Brain dysfunction changes in VCI patients mainly occur in brain regions associated with DMN. FC changes are significant between different brain regions, and the global topological organization is extensively damaged. However, current rs-fMRI studies on VCI are in their infancy, and relatively few studies have been reported. There are many analytical methods for rs-fMRI data, and data processing procedures are complex. Current studies have not only utilized inconsistent research methods but also inconsistent imaging parameters. In future studies, rs-fMRI analysis should be standardized. In addition, rs-fMRI combined with other fMRI techniques and neuropsychological assessments can provide more complete data information. With the advancement of technology and in-depth studies, functional imaging markers will play a more important role in the pathogenesis, early diagnosis and differential diagnosis, early intervention, and prognostic assessment in VCI and post-mTBI CI.

AUTHOR CONTRIBUTIONS

RW, NL, Y-YT, X-QG, JZ, and CY contributed to the literature search and manuscript preparation. RW, NL, and LY revised the manuscript. X-MZ designed the research. All authors read and approved the final manuscript.

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Herbal Medicine for Traumatic Brain Injury: A Systematic Review and Meta-Analysis of Randomized Controlled Trials and Limitations

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Background: This systematic review aimed to evaluate the effectiveness (functional outcomes and clinical symptoms) and safety (incidence of adverse events) of herbal medicine (HM) as monotherapy or adjunctive therapy to conventional treatment (CT) for traumatic brain injury (TBI).

Methods: We comprehensively searched 14 databases from their inception until July 2019. Randomized controlled trials (RCTs) using HM as monotherapy or adjunctive therapy to treat TBI patients were included. The primary outcome was functional outcomes, consciousness state, morbidity, and mortality. Meta-analysis was performed to calculate a risk ratio (RR) or mean difference (MD) with 95% confidence intervals (CIs), when appropriate data were available. Methodological quality of RCTs and the strength of evidence were also assessed.

Results: Thirty-seven RCTs with 3,374 participants were included. According to meta-analysis, HM as a monotherapy (RR 1.29, 95% CI: 1.21–1.37) or an adjunctive therapy to CT (RR 1.21, 95% CI: 1.16–1.27) showed significantly better total effective rate based on clinical symptoms, compared to CT alone. Subgroup analysis showed that HM had significantly improved post-concussion syndrome, dizziness, headache, epilepsy, and mild TBI, but not traumatic brain edema, compared to CT. Moreover, HM combined with CT had significantly improved post-concussion syndrome, mental disorder, headache, epilepsy, and mild TBI-like symptoms, but not cognitive dysfunction and posttraumatic hydrocephalus, compared to CT alone. When HM was combined with CT, functional outcomes such as activities of daily living and neurological function were significantly better than in patients treated using CT alone. In terms of the incidence of adverse events, HM did not differ from either CT (RR 0.88, 95% CI: 0.33–2.30) or placebo (RR 2.29, 95% CI: 0.83–6.32). However, HM combined with CT showed better safety profile than CT alone (RR 0.64, 95% CI: 0.44–0.93). Most studies had a high risk of

performance bias, and the quality of evidence was mostly rated "very low" to "moderate," mostly because the included studies had a high risk of bias and imprecise quantitative synthesis results.

Conclusion: The current evidence suggests that there is insufficient evidence for recommending HM for TBI in clinical practice. Therefore, further larger, high-quality, rigorous RCTs should be conducted.

Keywords: herbal medicine, traumatic brain injuries, systematic review, East Asian traditional medicine, post-concussion syndrome

INTRODUCTION

External force to the head can cause varying degrees of organic and/or functional abnormalities in the brain, ranging from mild to fatal. Traumatic brain injury (TBI) can be defined as "an alteration in brain function, or other evidence of brain pathology, caused by an external force" (1). TBI is a major threat to public health worldwide. In particular, this condition is an important cause of death and hospitalization (2). According to data from the Centers for Disease Control and Prevention (CDC) (3), the most common external causes of TBI are falls (common in childhood and in the elderly) and road traffic accidents (common in young adults). These results were confirmed in epidemiological studies carried out in Europe (2, 4). A recent systematic review of 82 population-based studies reporting the worldwide prevalence of TBI concluded that approximately 300 cases per 100,000 people occur per year, especially in Asia, with about 380 cases per 100,000, which is higher than the worldwide average (5).

Depending on the area and severity of the initial trauma, the severity of TBI can vary and is classified as mild, moderate, or severe using tools like the Glasgow Coma Scale (GCS) (6), which is based on the patient's state of consciousness (6). Many patients with TBI, even mild TBI, experience post-concussion syndrome (PCS), which involves a complex of symptoms including headache, dizziness, cognitive impairment, and neuropsychiatric symptoms (7). Moreover, TBI can cause persistent, sometimes life-long consequences, even in moderate or mild cases, and it can be associated with long-term negative outcomes that markedly reduce quality of life (QoL) of survivors, such as excess mortality, vegetative state, physical disability, cognitive impairment, depression, anxiety, psychosis, and seizures (8). In addition, TBI may be related to neurodegenerative diseases such as dementia (9), but not Parkinson's disease (10).

According to the CDC report (3), nearly half of patients with moderate-to-severe TBI undergoing inpatient rehabilitation experience pathological changes in their cognitive function between 1 and 5 years after injury (11). Therefore, to prevent long-term negative consequences and improve QoL, TBI requires long-term management as well as acute, post-injury treatment.

Complementary and integrative medicine (CIM) approaches, including acupuncture and herbal medicine (HM), are often used to supplement the limitations of conventional medicine (12, 13), improve effectiveness, and sometimes reduce side effects, even in the management of TBI (14, 15). In particular, HM has been used to manage brain trauma such as hemorrhage-related hydrocephalus (16), as well as long-term neurological diseases such as stroke (17), cerebral palsy (18), Parkinson's disease (19), vascular dementia (20), and Alzheimer's disease (21). In the field of brain trauma, common HMs such as *Goreisan* have been shown to prevent chronic subdural hematoma recurrence (22, 23), and the mechanism may involve the regulation of aquaporin, a water channel (24–26). Similarly, some HMs such as *Yokukansan* (27) and *Xuefu Zhuyu* decoction (28) have beneficial effects on TBI-related behavioral changes or cognitive impairment. In the management of TBI, HMs may have beneficial effects through complex mechanisms; they may reduce tumor necrosis factor-α or nitric oxide expression, improve blood-brain-barrier permeability, and reduce brain water content (29). However, no studies have yet synthesized all the clinical evidence for the effectiveness and safety of HM as an adjunctive or alternative therapy for various outcomes of TBI, including functional outcomes (mobility and global disability), mortality, quality of life, global clinical improvement, and adverse events. The present systematic review aimed to evaluate the effectiveness and safety of HM on these outcomes in TBI compared to placebo, no treatment, and conventional treatment (CT), to inform clinicians, policy makers, and patients in how to manage this disease.

Abbreviations: AEs, adverse events; AMED, the Allied and Complementary Medicine Database; BI, Barthel index; CDC, the Centers for Disease Control and Prevention; CENTRAL, the Cochrane Central Register of Controlled Trials; CIM, complementary and integrative medicine; CINAHL, the Cumulative Index to Nursing and Allied Health Literature; Cis, confidence intervals; CNKI, China National Knowledge Infrastructure; CT, conventional treatment; GCS, the Glasgow Coma Scale; GOS, Glasgow outcome scale; HM, herbal medicine; IRB, institutional review board; KCI, Korea Citation Index; KISS, Korean studies Information Service System; KMbase, Korean Medical Database; MD, mean difference; NIHSS, the National Institute Of Health Stroke Scale; OASIS, Oriental Medicine Advanced Searching Integrated System; PCS, post-concussion syndrome; PRISMA, the Preferred Reporting Items for Systematic Reviews and Meta-Analyses; QoL, quality of life; RCTs, randomized controlled trials; RISS, Research Information Service System; RR, risk ratio; SF-36, the 36-Item Short Form Health Survey; TBI, traumatic brain injury; TER, total effective rate; TESS, the treatment emergent symptom scale.

METHODS

Study Registration

The protocol of this systematic review has been published and registered in PROSPERO (registration number, CRD42018116559) (30), and the study was reported in

accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (31) and the Cochrane Handbook for Systematic Reviews of Interventions (32).

Data Sources and Search Strategy

As previously described, the following 14 databases were searched comprehensively: five English-language databases (Medline via PubMed, EMBASE via Elsevier, the Cochrane Central Register of Controlled Trials [CENTRAL], the Allied and Complementary Medicine Database [AMED] via EBSCO, and the Cumulative Index to Nursing and Allied Health Literature [CINAHL] via EBSCO), five Korean-language databases (Oriental Medicine Advanced Searching Integrated System [OASIS], Korean studies Information Service System [KISS], Research Information Service System [RISS], Korean Medical Database [KMbase], and Korea Citation Index [KCI]), three Chinese-language databases (China National Knowledge Infrastructure [CNKI], Wanfang Data, and VIP), and one Japanese database (CiNii). The initial search date was December 2, 2018 and we conducted an updated search on July 27, 2019 to retrieve more up-to-date and comprehensive evidence. Additionally, we searched the reference lists of the relevant articles and performed a manual search on Google Scholar to identify further eligible studies. We also included "gray literature," such as degree theses and conference proceedings, as well as the literature published in journals. There was no restriction on language, publication date, or publication status. The search strategies for all databases are available in **Supplemental Digital Content 1**.

Inclusion Criteria

Types of Studies

We included randomized controlled trials (RCTs) and excluded quasi-RCTs that used an inappropriate randomization method such as alternate allocation or allocation by birth date. Studies were excluded if they used the term "randomization" (随机) but failed to detail the randomization methods used. We included both parallel and crossover studies. Other study designs, such as *in vivo*, *in vitro*, case reports, and retrospective studies were excluded.

Types of Participants

We included studies involving patients diagnosed with TBI through medical or radiological examination, regardless of target symptoms, disease severity, sex, age, or race. We included all studies involving TBI patients, even if the diagnostic method of TBI was not clearly stated. We excluded studies that included participants with drug allergies or other serious medical conditions, such as cancer, liver disease, or kidney disease.

Types of Interventions

We included studies that used HM as a treatment intervention, regardless of which formulation of HM was used (e.g., decoction, tablets, capsules, pills, powders, and extracts); however, we only included studies in which HM was administered orally. We excluded studies that failed to detail the composition of the HM used, except when patent medicines were used whose composition could be found by searching the Internet. Studies comparing different types of HM were excluded. As control interventions, we included placebo, no treatment, and CT including surgery, medication, rehabilitation treatment, and psychotherapy for acute management and rehabilitation, which are baseline treatments for TBI. In the present study, acute management was defined as any treatment administered to stabilize the patients immediately after the injury (within 1 month). Rehabilitation was defined as any treatment of long-term impairments that aimed to restore to their previous level of health and was administered more than 1 month after injury (33). We included studies that combined HM with other therapies if the other therapies were used equally in both the treatment and control groups.

Types of Outcome Measures

The primary outcome measure was functional outcome, measured using the following validated scales: Barthel index (BI) (34), functional independence measurement (35), Fugl–Meyer assessment (36), and Glasgow Outcome Scale (GOS) (37). We also analyzed consciousness state measured using validated scales such as the GCS (38), with morbidity and mortality as primary outcome measures.

The secondary outcome measures were QoL, measured using validated assessment tools such as the 36-Item Short Form Health Survey (SF-36) (39), and adverse events (AEs), measured using the Treatment Emergent Symptom Scale (TESS) (40) or the incidence. We also analyzed the total effective rate (TER) as a secondary outcome; this is a non-validated outcome measure that is processed secondarily using certain evaluation criteria, such as improvement in clinical symptoms based on clinician ratings. In TER assessment, participants are generally classified as "cured" (痊愈), "markedly improved" (顯效), "improved" (有效), or "non-responsive" (無效) after treatment. The TER is calculated using the following formula: $TER = N1 + N2 + N3/N$, where $N1$, $N2$, $N3$, are the number of patients who are cured, markedly improved, and improved, respectively, while N is the total sample size. This outcome was considered a secondary outcome in this review as it lacks a unified standard and can be potentially heterogeneous.

Study Selection

As previously reported, two researchers (B. Lee and C-Y Kwon) independently selected the studies according to the above inclusion criteria. After removing duplicates, we screened the titles and abstracts of the retrieved studies for relevance; we then evaluated the full texts of the selected studies for final inclusion. Any disagreement was resolved through discussion with the other authors.

Data Extraction

Using a standardized data collection form in Excel 2007 (Microsoft, Redmond, WA, USA), two researchers (B. Lee and C-Y Kwon) independently extracted and double-checked the data from the included studies. Discrepancies were resolved through discussion with the other authors.

Using a predefined data collection form, we extracted information regarding the first author's name, publication year,

country, institutional review board (IRB), informed consent, sample size, and number of dropouts, diagnostic criteria, participant details, intervention, comparisons, duration of intervention and follow-up, outcome measures, outcomes, and AEs. We also extracted details of the HM used, including the name, source, dosage form, and dosage of each medical substance, as well as the principles, rationale, and interpretation of the intervention in terms of the Consolidated Standards of Reporting Trials Extension for Chinese Herbal Medicine Formulas 2017 (41). If the data were insufficient or ambiguous, we contacted the corresponding authors of the included studies via e-mail to request additional information.

Quality Assessment

As previously reported, two researchers (B. Lee and C-Y Kwon) independently evaluated the risk of bias of the included studies and the quality of evidence of the main findings. We resolved discrepancies through discussion with other researchers.

We assessed the methodological quality of the included studies using the Cochrane Collaboration's risk of bias tool (42). The following items were evaluated as either "low risk," "unclear," or "high risk": (1) random sequence generation, (2) allocation concealment, (3) blinding of participants and personnel, (4) blinding of outcome assessment, (5) completeness of outcome data, (5) selective reporting, and (6) other biases. In particular, we assessed other bias categories with an emphasis on baseline imbalance between the treatment and control groups in terms of participant characteristics such as mean age, sex, or disease severity, because baseline imbalance in factors that are strongly related to outcome measures can cause bias when estimating the intervention effect.

The quality of evidence for each main finding was assessed using the Grading of Recommendations Assessment, Development, and Evaluation approach (43), which uses the online program GRADEpro (https://gradepro.org/). The following items were evaluated as either "very low," "low," "moderate," or "high": risk of bias, inconsistency, indirectness, and imprecision of the results, and probability of publication bias.

Data Synthesis and Analysis

As previously described, we conducted descriptive analyses of the participants' details, interventions, and outcomes for all included studies. Using Review Manager version 5.3 software (Cochrane, London, UK), a meta-analysis was performed across studies that used the same types of intervention, comparison, and outcome measure. We pooled the dichotomous data using the risk ratio (RR) with 95% confidence intervals (CIs) and the continuous data using the mean difference (MD) with 95% CIs. We assessed clinical heterogeneity by comparing the distribution of important participant factors, such as age, sex, disease severity, and specific types of TBI, and we compared intervention factors such as co-interventions and control interventions among the included studies. Furthermore, statistical heterogeneity between the studies was assessed using both the chi-squared test and the I^2 statistic; $I^2 \geq 50\%$ indicated substantial heterogeneity, while those $\geq$75% indicated high heterogeneity. In the meta-analyses, a random-effects model was used when the heterogeneity was significant ($I^2 \geq 50\%$), while a fixed-effects model was used when the heterogeneity was not significant or when the number of studies included in the meta-analysis was <5, where estimates of inter-study variance have poor accuracy (44, 45). If the necessary data were available, we performed subgroup analyses to explain the heterogeneity or to assess whether the treatment effects varied between subgroups categorized according to the following criteria: (1) objective of interventions, such as acute management or rehabilitation, assessed in terms of time frame following injury; (2) severity of TBI, and (3) target symptoms, such as headache, dizziness, cognitive disorder, or mental disorder. To ascertain the robustness of the meta-analysis result, we conducted a sensitivity analyses by excluding (1) studies with a high risk of bias and (2) outliers that were numerically distant from the rest of the data.

Reporting Bias

We assessed reporting biases, such as publication bias, using funnel plots if more than 10 studies were included in the meta-analysis.

RESULTS

Study Description

We identified 27,258 studies through database searching and one study from the references of the relevant studies. After removing duplicated studies, we considered 626 studies relevant after screening of the titles and abstracts. Among these, we finally included 37 studies with 3,374 participants (46–82) in the qualitative synthesis, and 33 studies with 3,000 participants (46–48, 50, 51, 53–59, 61–74, 76–82) in meta-analysis after screening of the full-text articles (**Figure 1**).

We have summarized the general characteristics of the included studies in **Table 1**. One study was conducted in New Zealand (46) and all others were conducted in China. The median sample size of the included studies was 80 participants (range: 30–300 participants), meanwhile, the median treatment period was 5 weeks (range: 3 days to 18 months). Eighteen studies (46, 49–51, 53, 57, 60, 63, 67, 68, 70–72, 76–80) reported the disease period of the participants; three of these (50, 68, 80) conducted treatment for acute management (from onset of injury to 1 month post-injury), while 11 (49, 51, 53, 57, 60, 63, 67, 70, 72, 77, 79) reported rehabilitation-focused treatment (>1 month post-injury). With regards to the specific symptoms treated, the included studies recruited patients with PCS (12 studies) (48, 49, 51, 54, 55, 57, 59, 60, 63, 78, 79, 82), mental disorder (four studies) (53, 62, 64, 66), cognitive dysfunction (four studies) (46, 61, 68, 76), epilepsy (four studies) (67, 70–72), mild TBI (four studies) (73–75, 80), headache (three studies) (50, 56, 81), dizziness (two studies) (47, 65), brain edema (one study) (58), and hydrocephalus (77).

Seven studies recruited participants based on pattern identification (an approach of some East-Asian traditional medicines, including traditional Chinese medicine, which enables individual treatment by categorizing the signs and symptoms of patients into a series of syndrome concepts): five based on "blood stasis" (55, 57, 60, 63, 77), two on "phlegm" (48, 77), and one on "liver qi depression, blood deficiency, and

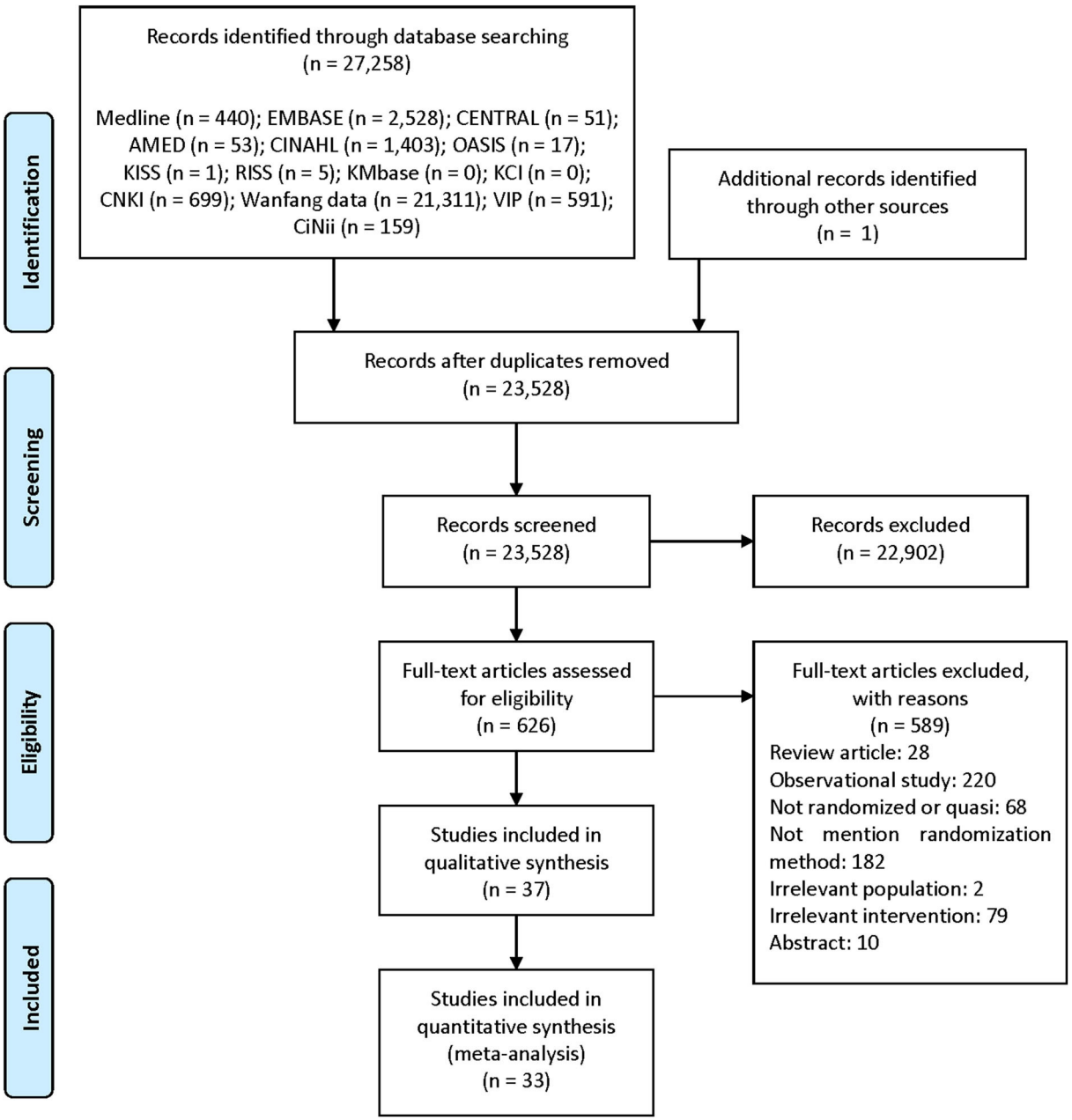

FIGURE 1 | PRISMA flow diagram of the literature screening and selection processes Moher et al. (83). AMED, Allied and Complementary Medicine Database; CENTRAL, Cochrane Central Register of Controlled Trials; CINAHL, Cumulative Index to Nursing and Allied Health Literature; CNKI, China National Knowledge Infrastructure; KCI, Korea Citation Index; KISS, Koreanstudies Information Service System; KMbase, Korean Medical Database; OASIS, Oriental Medicine Advanced Searching Integrated System; RISS, Research Information Service System.

spleen weakness" (79). Eleven studies compared HM with CT (47–49, 54, 58, 59, 65, 67, 74, 78, 81), three compared HM with a placebo (46, 68, 69), and 23 compared HM plus CT with CT alone (50–53, 55–57, 61, 70, 73, 75–77, 79, 80, 82). The CTs included symptomatic treatment, routine rehabilitation care, psychotherapy, and Western medication. Nine studies (46, 49, 56, 58, 61, 67, 71, 77, 81) conducted follow-up after treatment, with the range of follow-up periods being 1 month to 1 year. Various outcome measures were used depending on the target population, with the most frequently used outcome being TER, assessed in 29 studies (46–51, 53–60, 62, 63, 65, 67, 70, 72–74, 76–82). Ten studies (46, 50, 52, 56, 57, 59, 64, 69, 76, 77) reported IRB

TABLE 1 | General characteristics of the included studies.

Study ID	Sample size (included→ analyzed)	Mean age (range; year)	Sex (M:F)	Disease period (mean interval between TBI and study enrollment)	Population	Pattern identification	(A) Experimental intervention	(B) Control intervention	Treatment period/F/U	Outcome	Results+	Adverse events
(75)	88(44:44)→ 88(44:44)	(A) 32.4 (14–53) (B) 33.4 (16–56)	(A) 31:13 (B) 34:10	NR	Mild TBI (No abnormalities in the CT, MRI, and nervous system examination)	NR	(B) + HM	Symptomatic treatment	5 d/NR	1. Clinical memory scale	1. (A)>(B)* (all)#	NR
(73)	84(42:42)→ 84(42:42)	(A) 36.8 ± 5.2 (15–55) (B) 37.2 ± 4.9 (16–58)	(A) 24:18 (B) 26:16	NR	Mild TBI–like symptoms (GCS ≥ 13, no abnormalities in CT)	NR	(B) + HM	Symptomatic treatment, bed rest, Nimodipine 30 mg tid	20 d/NR	1. Mean blood flow velocity of middle cerebral artery and basilar artery (Doppler flowmetry) 2. Clinical symptom relief time 3. TER (clinical symptom)	1. (A)<(B)* (all) 2. (A)<(B)* (all) 3. (A)>(B)*	None
(74)	80(40:40)→ 80(40:40)	(A) 42 ± 9.8 (B) 40 ± 8.1	(A) 30:10 (B) 32:8	NR	Mild TBI (No abnormalities in vital sign and CT)	NR	HM, symptomatic treatment, bed rest	Symptomatic treatment, bed rest, Citicoline sodium 0.5 g plus 0.9% sodium chloride IV inj. qd	3–7 d/NR	1. TER (clinical symptom)	1. (A)>(B)+	NR
(47)	30(15:15)→ 30(15:15)	(A) 42.3 ± 1.2 (B) 42.0 ± 1.9	(A) 10:5 (B) 9:6	NR	Dizziness	NR	HM	Nimodipine 30 mg tid	5 d/NR	1. TER (clinical symptom)	1. (A)>(B)*	NR
(50)	62(31:31)→ 62(31:31)	(A) 38.7 ± 10.3 (20–62) (B) 38.3 ± 10.2 (19–61)	(A) 18:13 (B) 17:14	(A) 9.02 ± 2.16 d (2–16) (B) 9.57 ± 2.45 d (3–18)	Mild TBI induced headache	NR	(B) + HM	Symptomatic treatment, bed rest	NR/NR	1. TER (clinical symptom) 2. recurrence rate	1. (A)>(B)* 2. (A)<(B)*	NR
(48)	156(78:78)→ 156(78:78)	(A) 53.4 ± 8.2 (26–69) (B) 53.1 ± 8.2 (29–67)	(A) 25:53 (B) 24:54	NR	PCS (No abnormalities in the CT, MRI, CSF and nervous system examination)	Phlegm turbidity middle obstruction	HM	symptomatic treatment, psychotherapy	6 week/NR	1. TER (clinical symptom) 2. VAS (according to pattern identification)	1. (A)>(B)* 2. (A)<(B)+ (all)	Mild transaminase elevation (A) 2, (B) 3; WBC elevation (A) 2, (B) 1; mild memory impairment (A) 3, (B) 4
(80)	80(40:40)→ 80(40:40)	(A) 38.5 (12–60) (B) 40.5 (13–58)	(A) 22:18 (B) 26:14	(A) Mean 4.5 h (35 min−8 h) (B) Mean 5.2 h (45 min−7 h)	Mild TBI–like symptoms (GCS ≥ 13, no abnormalities in the CT)	NR	(B) + HM	Nimodipine 30 mg tid	20 d/NR	1. Mean blood flow velocity of middle cerebral artery and basilar artery (Doppler flowmetry) 2. TER (clinical symptom) 3. Number of people with clinical symptom relief	1. N.S (1 d after treatment), (A)<(B)* (7, 14 and 20 d after treatment) 2. (A)>(B)* 3. (A)>(B)*	None

(Continued)

TABLE 1 | Continued

Study ID	Sample size (included→ analyzed)	Mean age (range; year)	Sex (M:F)	Disease period (mean interval between TBI and study enrollment)	Population	Pattern identification	(A) Experimental intervention	(B) Control intervention	Treatment period/F/U	Outcome	Results	Adverse events
(79)	60(30:30)→ 60(30:30)	(A) 40.8 ± 10.3 (B) 41.3 ± 10.3	(A) 20:10 (B) 17:13	(A) 23.63 ± 13.58 mo (B) 24.65 ± 15.21 mo	PCS (No abnormalities in the CT, MRI, CSF, and nervous system examination)	Liver qi depression, blood deficiency, and spleen weakness	(B) + HM	Symptomatic treatment	6 wk/NR	1. TER (Rivermead post–concussion symptoms questionnaire score) 2. SF−36	1. (A)>(B)+ 2. (A)>(B)* (vitality, social functioning, role limitations due to emotional problems and mental health), (A)>(B)+ (mental component summary), N.S (others)	None
(81)	300(150:150)→ 300(150:150)	37.4	155:145	NR	Headache (No abnormalities in CT)	NR	HM	Analegics	2 mo/1 mo	1. TER (BRS−6) at 1 mo f/u 2. TER (headache)	1. (A)>(B)+ 2. (A)>(B)+	NR
(82)	124(62:62)→ 124(62:62)	(A) 40.5 ± 5.5 (B) 41.2 ± 5.3	(A) 36:26 (B) 38:24	NR	PCS (No abnormalities in the CT and nervous system examination)	NR	(B) + HM	Piracetam 0.4 g tid, Oryzanol 10 mg tid, Nimodipine 20 mg tid, Nicergoline 20 mg tid, psychotherapy	4 wk/NR	1. TER (clinical symptom) 2. ADL 3. WMS	1. (A)>(B)+ 2. (A)<(B)+ 3. (A)>(B)+	None
(52)	120(60:60)→ 120(60:60)	52.4 ± 10.4 (20–76)	82:38	NR	TBI (Mild TBI 53, SAH 29, brain contusion 18, subdural/epidural hematoma 20)	NR	(B) + HM	Conventional nutritional nerves and improved microcirculation therapy	NR/NR	1. Clinical symptom relief time 2. Coagulation items (plasmin prothrombin time, activity, activated paial prothrombin time, fibrinogen, thrombin time), platelet count, residual bleeding/total bleeding	1. (A)<(B)+ 2. (A)<(B)* (residual bleeding/total bleeding), N.S (others)	NR
(55)	99(51:48)→ 99(51:48)	(A) 45.6 ± 8.7 (18–66) (B) 43.96 ± 11.10 (17–65)	(A) 33:18 (B) 29:19	NR	PCS	Stasis and stagnation of qi and blood	(B) + HM	Psychotherapy, physical therapy, vitamin B, Oryzanol	2 wk/NR	1. TER (clinical symptom)	1. (A)>(B)+	None

(Continued)

TABLE 1 | Continued

Study ID	Sample size (included→ analyzed)	Mean age (range; year)	Sex (M:F)	Disease period (mean interval between TBI and study enrollment)	Population	Pattern identification	(A) Experimental intervention	(B) Control intervention	Treatment period/F/U	Outcome	Results	Adverse events
(56)	96(48:48)→ 96(48:48)	(A) 41 ± 5.8 (17–64) (B) 41 ± 4.6 (17–64)	(A) 30:18 (B) 29:19	NR	Headache (No abnormalities in CT or MRI)	NR	(B) + HM	Nimodipine 60 mg tid, Piracetam 0.8 g tid, symptomatic treatment	21 d/3 mo	1. TER (clinical symptom) 2. Headache symptom improvement time 3. recurrence rate (3 mo)	1. (A)>(B)* 2. (A)<(B)* 3. (A)<(B)*	(A) GI discomfort 1 (B) dizziness and mild nausea 2
(57)	60(30:30)→ 60(30:30)	(A) 47.1 ± 6.4 (12–79) (B) 48.2 ± 11.3 (13–81)	(A) 18:12 (B) 17:13	(A) 12.03 ± 4.01 mo (6–18) (B) 12.15 ± 3.76 mo (6–18)	PCS (No abnormalities in CT, CSF and nervous system examination, no mental abnormalities)	Obstruction of clear orifices and blood stasis	(B) + HM	Symptomatic treatment, HBOT (once a day, total 30 times)	6–18 mo/NR	1. TCM syndrome score 2. Peak velocity and end–diastolic velocity of bilateral vertebral artery and basilar artery (Doppler flowmetry) 3. TER (clinical symptom, TCM syndrome score)	1. (A)<(B)+ 2. (A)>(B)* (peak velocity of left vertebral artery and end–diastolic velocity of basilar artery), (A)>(B)+ (others) 3. (A)>(B)*	NR
(54)	100(60:40)→ 100(60:40)	(A) 43.5 (B) 42.0	(A) 36:24 (B) 28:12	NR	PCS	NR	HM	Pyritinol hydrochloride 0.2 g tid	5 wk/NR	1. TER (clinical symptom)	1. (A)>(B)*	NR
(53)	80(40:40)→ 80(40:40)	(A) 16–70 (B) 17–69	(A) 23:17 (B) 22:18	(A) 1–7 yr (B) 1–6.8 yr	Mental disorder (CCMD−3, HAMA≥14, HAMD≥17)	NR	(B) + HM	Fluoxetine 20 mg qd	8 wk/NR	1. TER (HAMD, HAMA, TESS) 2. HAMD 3. HAMA	1. (A)>(B)* 2. (A)<(B)* 3. (A)<(B)*	NR
(58)	40(20:20)→ 40(20:20)	(A) 43.1 ± 17.7 (B) 47.8 ± 19.2	(A) 14:6 (B) 13:7	NR	Traumatic brain edema (GCS 9–15)	NR	HM	20% mannitol 125 ml IV inj.	14 d/1 mo	1. GCS 2. Intracranial pressure (mmH_2O) 3. China stroke scale 4. Serum CRP concentration 5. Serum Na+ concentration 6. Serum K+ concentration 7. TER (TCM syndrome) 8. TER (clinical symptom) 9. TER (CT findings)	1. N.S 2. N.S 3. N.S 4. N.S 5. (A)>(B)+ 6. (A)>(B)+ 7. N.S 8. N.S 9. N.S	None

(Continued)

TABLE 1 | Continued

Study ID	Sample size (included→ analyzed)	Mean age (range; year)	Sex (M:F)	Disease period (mean interval between TBI and study enrollment)	Population	Pattern identification	(A) Experimental intervention	(B) Control intervention	Treatment period/F/U	Outcome	Results	Adverse events
(75)	60(31:29)→ 60(31:29)	(A) 35.8 ± 12.6 (B) 37.7 ± 19.9	(A) 19:12 (B) 18:11	(A) 15.10 ± 3.75 d (B) 16.50 ± 4.79 d	Cognitive dysfunction (3<GCS≤8)	NR	(B) + HM	Symptomatic treatment	54 d/NR	1. TER (Rancho Los Amigos levels of cognitive functioning scale) 2. Serum levels of NSE and S100β	1. (A)>(B)* 2. (A)<(B)* (all)	None
(77)	60(30:30)→ 60(30:30)	(A) 47.1 ± 6.6 (35–66) (B) 46.7 ± 6.4 (37–64)	(A) 16:14 (B) 18:12	(A) 5.96 ± 0.81 mo (3–11) (B) 5.68 ± 0.76 mo (3–10)	Posttraumatic hydrocephalus	Phlegm and blood stasis obstructing the collaterals	(B) + HM	20% mannitol 125–250 ml IV inj. bid, acetazolamide 0.25 g bid–tid	15 d/1 mo	1. Serum levels of MBP, S100β, and p73 factor 2. NIHSS 3. BI 4. TCM syndrome scores 5. TER (clinical symptom and sign, degree of hydrocephalus, and TCM syndrome score) 6. Degree of hydrocephalus (f/u 1 mo)	1. (A)<(B)+ (MBP, S100β), N.S (p73 factor) 2. (A)<(B)+ 3. (A)>(B)+ 4. (A)<(B)+ 5. (A)>(B)* 6. (A)<(B)*	None
(49)	80(40:40)→ 80(40:40)	(A) 56.8 ± 12.3 (37–79) (B) 56.9 ± 10.8 (38–74)	(A) 21:19 (B) 22:18	(A) 1.2 ± 0.4 yr (0.4–1.8) (B) 1.1 ± 0.3 yr (0.3–1.6)	PCS (No abnormalities in CT and neurological examination)	NR	HM	Citicoline 0.5 g plus 10% glucose 200 ml IV inj. qd, Piracetam 0.8 g tid, Oryzanol 20 mg tid	2 mo/1 yr	1. TER (TCM syndrome)	1. (A)>(B)*	NR
(61)	70(35:35)→ 70(35:35)	(A) 47.1 ± 14.3 (21–70) (B) 48.3 ± 15.3 (19–72)	(A) 26:9 (B) 28:7	NR	Cognitive dysfunction (MMSE<24, GCS 13–15)	NR	(B) + HM	Neurosurgery conventional treatment	1 mo/6 mo	1. MMSE 2. computer–aided cognitive measurement system	1. (A)>(B)* (1 mo after treatment), (A)<(B)+ (f/u 6 mo) 2. (A)>(B)+ (1 mo, f/u 6 mo)	None
(51)	200(100:100)→ 189(96:93)	(A) 34.2 ± 7.1 (B) 32.4 ± 6.7	(A) 64:32 (B) 64:32	(A) 7.55 ± 2.60 mo (B) 7.55 ± 3.17 mo	PCS (No abnormalities in the CT, MRI, CSF, and nervous system examination)	NR	(B) + HM	Psychological and behavioral therapy, symptomatic treatment, rehabilitation treatment	Until clinical symptoms disappeared for 2 wk or until 12 wk/NR	1. TER (clinical symptom) 2. Cure time	1. (A)>(B)+ 2. (A)<(B)*	(A) 4 (B) 2

(Continued)

TABLE 1 | Continued

Study ID	Sample size (included→ analyzed)	Mean age (range; year)	Sex (M:F)	Disease period (mean interval between TBI and study enrollment)	Population	Pattern identification	(A) Experimental intervention	(B) Control intervention	Treatment period/F/U	Outcome	Results	Adverse events
(46)	78(36:42)→ 53(25:28)	(A) 38.6 ± 14.1 (B) 38.4 ± 15.7	(A) 17:19 (B) 22:20	(A) Median 98 d (B) median 94.5 d	Cognitive dysfunction (cognitive failures questionnaire>30)	NR	HM	Placebo (dextrin and magnesium stearate)	6 mo/3 mo	1. CNS vital signs online neuropsychological test 2. Cognitive failures questionnaire 3. Rivermead postconcussion symptom questionnaire 4. Quality of life 5. Hospital anxiety and depression scale 6. Modified fatigue impact scale 7. Extended GOS	1. (A)<(B)* (complex attention, executive function), N.S (others) 2. N.S 3. N.S 4. N.S 5. N.S 6. N.S 7. N.S	(A) Headache 1, sore tongue 1, itchiness 1 (B) Difficulty sleeping 1, headache 1, itchiness 1, upset stomach 1, blood in urine 1
(68)	142(70:72)→ 130(65:65)	38.6 (6–69)	74:56	13 ± 6 d (7–21)	Memory impairment (WMS<100, no aphasia)	NR	HM	Placebo (amylum)	4 wk/NR	1. memory quotient (WMS)	1. (A)>(B)+	(A) Nausea 2, diarrhea 2, mild hypotension 4 (B) none
(69)	112(56:56)→ 112(56:56)	(A) 42.8 ± 5.1 (32–63) (B) 42.6 ± 5.1 (30–62)	(A) 36:20 (B) 33:23	NR	TBI	NR	HM	Placebo	8 wk/NR	1. Simple test for evaluating hand function 2. Fugi–Meyer assessment 3. Modified BI	1. (A)>(B)+ 2. (A)>(B)+ 3. (A)>(B)+	NR
(70)	68(34:34)→ 68(34:34)	(A) 37.5 ± 2.6 (13–61) (B) 36.8 ± 2.4 (14–62)	(A) 19:15 (B) 18:16	(A) 4.5 ± 1.3 yr (2–7) (B) 4.3 ± 1.1 yr (1–8)	Epilepsy	NR	(B) + HM	Carbamazepine 5–20 mg/(kg·d)	NR/NR	1. TER (clinical symptom)	1. (A)>(B)*	(A) GI symptom 6, dizziness 3, rash 2, hair loss 3 (B) GI symptom 5, dizziness 4, rash 3, hair loss 2
(66)	40(20:20)→ 40(20:20)	(A) 37.2 ± 3.5 (30–59) (B) 34.6 ± 5.7 (28–54)	(A) 12:8 (B) 14:6	NR	Mental disorder (CCMD-3)	NR	(B) + HM	Olanzapine 5–20 mg/d	8 wk/NR	1. PANSS 2. TESS	1. (A)<(B)* 2. N.S	(A) GI discomfort 1, dizziness 1, dry mouth 1 (B) GI discomfort 2, nausea and vomiting 1, drowsiness 1, constipation 1, dry mouth 1

(Continued)

TABLE 1 | Continued

Study ID	Sample size (included→ analyzed)	Mean age (range; year)	Sex (M:F)	Disease period (mean interval between TBI and study enrollment)	Population	Pattern identification	(A) Experimental intervention	(B) Control intervention	Treatment period/F/U	Outcome	Results	Adverse events
(67)	80(40:40)→ 80(40:40)	(A) 64.2 ± 4.4 (19–88) (B) 63.9 ± 4.6 (19–87)	(A) 26:14 (B) 28:12	(A) 2.4 ± 0.4 mo (1–13) (B) 2.7 ± 0.3 mo (1–15)	Epilepsy	NR	HM	Sodium valproate sustained release tablets 500 mg bid	3 mo/1 mo	1. TER (clinical symptom) 2. Number of seizures	1. (A)>(B)* 2. (A)<(B)*	None
(65)	96(48:48)→ 96(48:48)	(A) 36 (22–68) (B) 40 (20–82)	(A) 31:17 (B) 35:13	NR	Dizziness	NR	HM	Flunarizine 5 mg bid	7–20 d/NR	1. TER (clinical symptom)	1. (A)>(B)+	NR
(64)	108(54:54)→ 108(54:54)	(A) 58.0 ± 6.4 (B) 58.1 ± 6.9	(A) 32:22 (B) 30:24	NR	Mental disorder (CCMD-3)	NR	(B) + HM	Olanzapine 5–20 mg/d bid	8 wk/NR	1. PANSS 2. TESS 3. Brief psychiatric rating scale 4. GQOLI-74	1. (A)<(B)* 2. N.S 3. (A)<(B)* 4. (A)>(B)* (body health, psychological conditions, social function), N.S (others)	(A) Nausea and vomiting 2, dizziness 1, GI discomfort 1, dry mouth 1 (B) Nausea and vomiting 3, GI discomfort 2, drowsiness 1, constipation 1
(78)	78(43:35)→ 78(43:35)	(A) 39.2 ± 5.0 (18–58) (B) 38.7 ± 6.2 (20–63)	(A) 18:25 (B) 11:24	(A) 14.4 ± 4.5 mo (B) 16.8 ± 3.7 mo	PCS	NR	HM	Oryzanol tid	2 wk/NR	1. TER (clinical symptom)	1. (A)>(B)* (all)	NR
(60)	86(43:43)→ 86(43:43)	(A) 52.3 ± 10.2 (34–68) (B) 53.1 ± 10.2 (32–67)	(A) 22:21 (B) 19:24	(A) 20.59 ± 4.12 mo (2–36) (B) 18.26 ± 4.52 mo (3–36)	PCS	Blood stasis affecting the clear orifices	(B) + HM	HBOT (once a day, 5 times per week)	4 wk/NR	1. TCM syndrome score 2. TER (TCM syndrome score) 3. NIHSS 4. Mean blood flow velocity of bilateral vertebral artery and basilar artery (Doppler flowmetry)	1. (A)<(B)* 2. (A)>(B)* 3. (A)<(B)* 4. (A)<(B)* (all)	NR
(59)	50(25:25)→ 50(25:25)	(A) 45.2 ± 1.0 (30–60) (B) 46.2 ± 1.3 (31–60)	(A) 13:12 (B) 14:11	NR	PCS	NR	HM	Oryzanol 20 mg tid	NR/NR	1. TER (clinical symptom) 2. Symptom improvement time 3. Hospitalization time	1. (A)>(B)* 2. (A)<(B)* 3. (A)<(B)*	NR

(Continued)

TABLE 1 | Continued

Study ID	Sample size (included→ analyzed)	Mean age (range; year)	Sex (M:F)	Disease period (mean interval between TBI and study enrollment)	Population	Pattern identification	(A) Experimental intervention	(B) Control intervention	Treatment period/F/U	Outcome	Results	Adverse events
(62)	48(24:24)→ 48(24:24)	(A) 34.5 ± 5.2 (28–52) (B) 35.1 ± 5.7 (30–54)	(A) 14:10 (B) 16:8	NR	Mental disorder	NR	(B) + HM	Olanzapine 5–20 mg/d	8 wk/NR	1. PANSS 2. TESS 3. TER (clinical symptom)	1. (A)<(B)+ 2. (A)<(B)+ 3. (A)>(B)*	(A) GI discomfort 1, dry mouth 1 (B) GI discomfort 2, constipation 2, dry mouth 2, drowsiness 2
(71)	60(30:30)→ 60(30:30)	(A) 31.5 ± 15.5 (B) 30.5 ± 13.7	(A) 26:4 (B) 25:5	(A) 6.2 ± 3.10 yr (B) 6.4 ± 2.9 yr	Epilepsy	NR	(B) + HM	Carbamazepine 0.1 g tid, γ-aminobutyric acid 1.5 g tid	2 mo/0.5 yr	1. TER (clinical symptom)	1. No statistical analysis	(A) rash 2, drowsiness 2, nausea 1 (B) leukopenia 4, rash 4, drowsiness 5, nausea 3
(72)	79(41:38)→ 79(41:38)	(A) 28–65 (B) 25–63	(A) 28:13 (B) 26:12	(A) NR (1 mo−3 yr) (B) NR (1 mo−2.5 yr)	Epilepsy	NR	(B) + HM	Sodium valproate sustained-release tablets 500 g bid	3 mo/NR	1. TER (clinical symptom)	1. (A)>(B)*	NR
(63)	120(60:60)→ 120(60:60)	(A) 50.6 ± 8.2 (B) 48.7 ± 9.1	(A) 36:24 (B) 34:26	(A) 12.47 ± 4.64 mo (B) 12.62 ± 4.96 mo	PCS (No abnormalities in CT)	blood stasis obstructing clear orifices and blood stasis	(B) + HM	Diclofenac sodium sustained release capsule 25 mg bid, Piracetam 0.8 g tid, Oryzanol 20 mg tid, HBOT (once a day)	1 mo/NR	1. TCM syndrome score 2. Mean blood flow velocity of bilateral vertebral artery and basilar artery (Doppler flowmetry) 3. TER (clinical symptom, TCM syndrome score)	1. (A)<(B)+ 2. (A)>(B)* (all) 3. (A)>(B)*	None

"" and "+" mean significant differences between two groups, $p < 0.05$ and $p < 0.01$, respectively. "N.S" means no significant difference between two groups, $p > 0.05$.*

 # "all" means that all of the subscales in the outcome measurement tool were improved.

ADL, activities of daily living; BI, barthel index; BRS-6, 6-point behavioral rating scale; CCMD, Chinese classification of mental disorders; CNS, central nervous system; CRP, C-reactive protein; CSF, cerebrospinal fluid; CT, computed tomography; F/U, follow up; GCS, Glasgow coma scale; GI, gastrointestinal; GOS, Glasgow outcome scale; GQOLI-74, generic quality of life inventory-74; HAMA, Hamilton anxiety rating scale; HAMD, Hamilton depression rating scale; HBOT, hyperbaric oxygen therapy; HM, herbal medicine; MBP, myelin basic protein; MMSE, mini-mental state examination; MRI, magnetic resonance imaging; NIHSS, national institute of health stroke scale; NR, not reported; NSE, neuron-specific enolase; PANSS, positive and negative symptoms scale; PCS, post-concussion syndrome; SAH, subarachnoid hemorrhage; SF-36, 36-item short form survey; TBI, traumatic brain injury; TCM, traditional Chinese medicine; TER, total effective rate; TESS, treatment emergent symptom scale; VAS, visual analog scale; WBC, white blood cell; WMS, Wechsler memory scale.

approval, and 20 (46, 48, 50–52, 56–61, 63, 64, 66, 69, 76–79, 82) reported that they had received consent from the participants.

The included studies used a variety of HMs, with the most common being *Xuefuzhuyu* decoction (six studies) (50, 60, 62, 66, 67, 72), followed by the patented drug *Yangxue Qingnao* granules (four studies) (68, 73, 80, 82). In total, 89 different herbs were used in the included studies, with the most frequently used being *Cnidii Rhizoma* (27 studies), followed by *Angelicae Gigantis Radix* (25 studies), *Persicae Semen* (19 studies), *Carthami Flos* (17 studies), *Bupleuri Radix* (16 studies), *Paeoniae Radix Rubra* (16 studies), and *Acori Graminei Rhizoma* (15 studies) (**Supplemental Digital Content 2**).

Risk of Bias

All the included studies reported appropriate random sequence generation methods; however, only two used a sealed opaque envelope (79) or independent allocation manager (46) to conceal allocation. Only one study (46) appropriately blinded both the participants and personnel, and two studies (68, 69) used placebo drugs as a control intervention but did not report appropriate blinding of personnel. None of the included studies reported blinding of the outcome assessor. Two studies (51, 68) that performed per-protocol analysis were assessed as having a high risk of attrition bias, while two (50, 51) that reported only TER, a secondary processed outcome without the raw data, were assessed as having a high risk of reporting bias. Thirty-five studies (46–51, 53–60, 62–82) reported no significant baseline difference in demographic data between the two groups, and were rated as having low risk of bias in the other potential sources of bias domains (**Figures 2**, **3**).

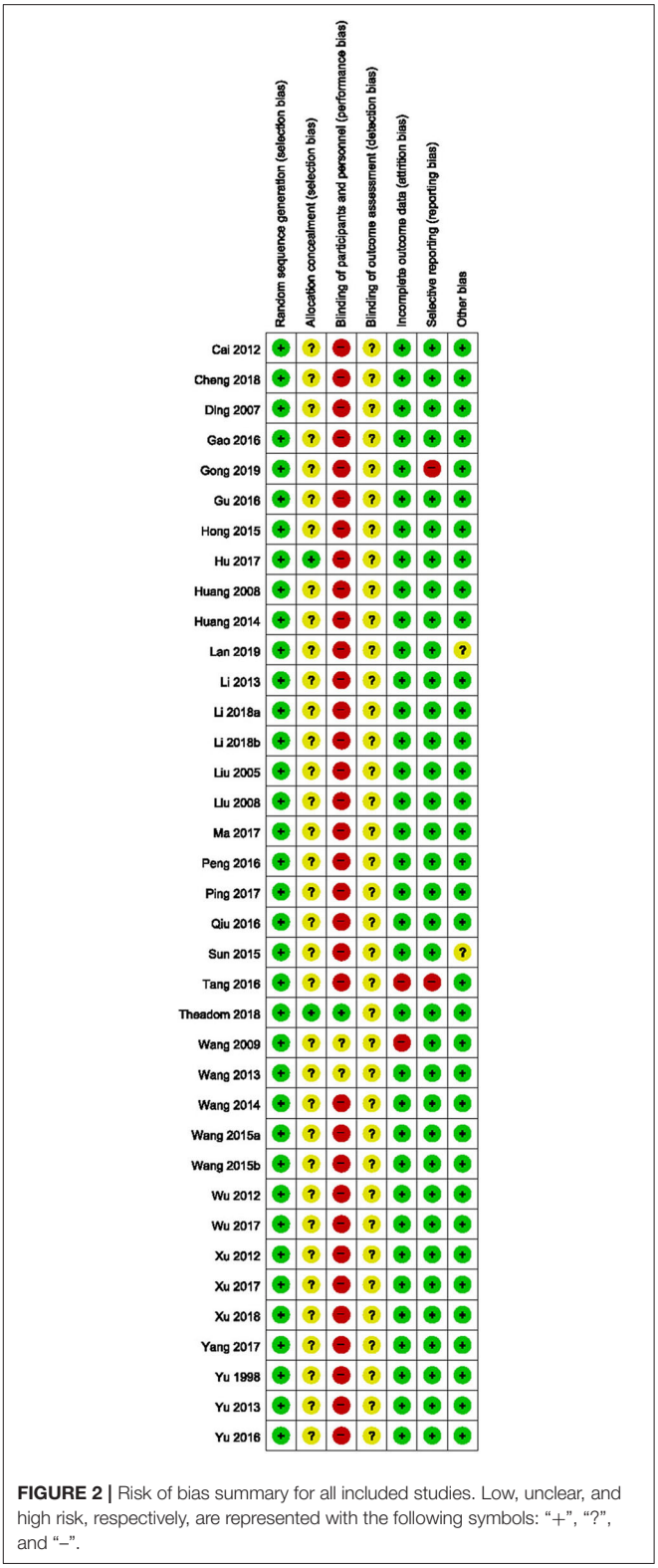

FIGURE 2 | Risk of bias summary for all included studies. Low, unclear, and high risk, respectively, are represented with the following symbols: "+", "?", and "–".

HM vs. CT

Effectiveness

Eleven studies (47–49, 54, 58, 59, 65, 67, 74, 78, 81) were included in the comparison of effectiveness: five (48, 49, 54, 59, 78) were conducted on patients with PCS, (47, 65) two on patients with dizziness, one each on patients with headache (81), epilepsy (67), mild TBI (74), and traumatic brain edema (58). Although there were no differences in the functional outcomes and states of consciousness between two groups, HM group showed significantly better outcomes in TER based on clinical symptoms, symptom improvement time, and duration of hospitalization.

In one study involving traumatic brain edema (58), the groups did not differ in terms of functional outcome, as measured using the GOS, after 1 month of post-intervention follow-up (MD: 0.10, 95% CI: −0.13 to 0.33), nor did they differ in terms of consciousness state, measured using the GCS after 14 days of intervention (MD: 0.05, 95% CI: −0.12–0.22). In addition, the two groups did not differ in terms of intracranial pressure or neurological function, measured using the China stroke scale after treatment. However, in 10 studies, the TER based on clinical symptoms was significantly improved in the HM group (RR: 1.29, 95% CI: 1.21–1.37, $I^2 = 0\%$). In a subgroup analysis based on the target symptoms of TBI, the HM group showed significantly better outcomes in patients with PCS, dizziness, headache, epilepsy, and mild TBI of all causes except traumatic brain edema (**Table 2**; **Figure 4**) (**Supplemental Digital Content 3**).

In a study by Xu et al. (59), when HM was administered to patients with PCS, the symptom improvement time and hospitalization time were significantly shorter than in the CT group ($P < 0.05$, all). Wang and Tian (67) reported that, when

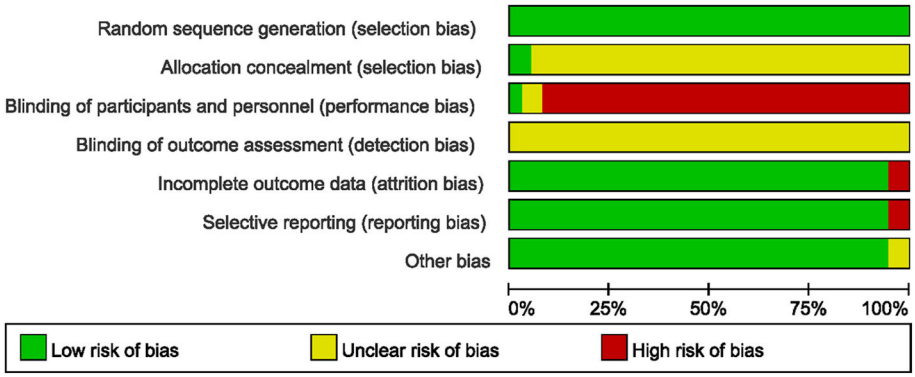

FIGURE 3 | Risk of bias graph for all included studies.

HM was administered to patients of epilepsy, the number of seizures was significantly lower than in the CT group ($P < 0.05$).

Safety

Three studies reported AEs during the intervention, and a meta-analysis of these showed no difference in the incidence of AEs between the two groups (RR: 0.88, 95% CI: 0.33–2.30; **Table 2**) (**Supplemental Digital Content 3**).

HM vs. Placebo

Efficacy

Three studies (46, 68, 69) compared HM with a placebo. Two of these (46, 68) were conducted on patients with cognitive dysfunction, while the other one (69) did not include participants with specific symptoms. Collectively, the functional outcomes showed inconsistent results between studies, and there was no significant difference in QoL between two groups. However, memory impairment was improved more in the HM group.

In a study by Wang (69), the HM group showed improved functional outcomes, as assessed using the Fugl–Meyer assessment (MD: 9.63, 95% CI: 8.21–11.05) and modified BI (MD: 18.54, 95% CI: 17.27–19.81), after 8 weeks of treatment. Additionally, hand function in the HM group was significantly better than in the placebo group ($P < 0.01$). After patients with cognitive dysfunction were treated ifor 6 months (46), physical disability was measured using the GOS and QoL measured by the QoL after brain injury scale showed no significant differences between the two groups (GOS: MD, 0.00; 95% CI: −4.17 to 4.17; QoL after brain injury scale: MD, 1.91; 95% CI: −9.58 to 13.40; **Table 2**) (**Supplemental Digital Content 3**). In addition, after intervention, there were no significant differences between the groups in terms of neurobehavioral sequelae, mood, or fatigue. However, complex attention and executive functioning in the HM group were significantly better than in the placebo group ($P < 0.05$). In a study by Wang et al. (68) involving patients with memory impairment, the HM group showed significantly better memory quotient, measured using the Wechsler Memory Scale, than the placebo group after 4 weeks of treatment ($P < 0.01$). The results of sensitivity analysis by excluding low quality studies (that had 4 or less low risk of bias on the seven domains of the risk of bias tool) were consistent in GOS and QoL (**Supplemental Digital Content 4**).

Safety

Two studies (46, 68) recruiting patients with cognitive dysfunction reported AEs during the treatment period. There was no difference in the incidence of AEs between the two groups (RR: 2.29, 95% CI: 0.83–6.32, and $I^2 = 79\%$; **Table 2**; **Figure 4**) (**Supplemental Digital Content 3**), nor was there any difference between the two groups in a sensitivity analysis that excluded studies with a high risk of bias (**Supplemental Digital Content 4**).

HM Plus CT vs. CT Alone

Effectiveness

Twenty-three studies (50–53, 55–57, 61, 70, 73, 75–77, 79, 80, 82) compared effectiveness between HM plus CT and CT alone. Seven of these (51, 55, 57, 60, 63, 79, 82) were conducted on patients with PCS, four (53, 62, 64, 66) on patients with mental disorder, three on patients with epilepsy (70–72), three on patients with mild TBI (73, 75, 80), two on patients with cognitive dysfunction (61, 76), two on patients with headache (50, 56), and one each on patients with hydrocephalus (77) and TBI (52). In summary, the function and TER of various symptoms were significantly improved when HM was added to CT. However, there were inconsistent results in QoL between studies.

Huang and Li (82) conducted 4 weeks of treatment in patients with PCS; they found that activities of daily living were significantly better in the HM plus CT group than in the CT alone group (MD: −3.30, 95% CI: −5.04 to −1.56). Ping (77) conducted 15 days of treatment in patients with post-traumatic hydrocephalus; their results showed that functional outcomes, as measured using BI, were significantly better in the HM group (MD: 11.14, 95% CI: 5.43–16.85) (**Table 2**) (**Supplemental Digital Content 3**). When HM was added to the CT, there was a significant difference in neurological function after treatment compared to that with CT alone, as measured using the National Institute of Health Stroke Scale (NIHSS) ($P < 0.01$), and degree of hydrocephalus differed significantly between

TABLE 2 | Summary of findings in all comparisons.

Outcomes		No. participants (RCTs)	Anticipated absolute effects (95% CI)		Relative effect (95% CI)	Quality of evidence (GRADE)	Comments
			Risk with control group	Risk with treatment group			
Herbal medicine vs. conventional treatment							
GOS	Total (traumatic brain edema)	40 (1)	–	MD 0.1 higher (0.13 lower to 0.33 higher)	–	⊕◯◯◯ VERY LOW	Risk of bias (−1) Imprecision (−2)
GCS	Total (traumatic brain edema)	40 (1)	–	MD 0.05 higher (0.12 lower to 0.22 higher)	–	⊕◯◯◯ VERY LOW	Risk of bias (−1) Imprecision (−2)
TER (clinical symptom)	Total	1,010 (10)	727 per 1,000	938 per 1,000 (880–996)	RR 1.29 (1.21–1.37)	⊕⊕◯◯ LOW	Risk of bias (−1) Indirectness (−1)
Subgroup (target symptom)	PCS	384 (4)	697 per 1,000	892 per 1,000 (801–996)	RR 1.28 (1.15–1.43)	⊕⊕◯◯ LOW	Risk of bias (−1) Indirectness (−1)
	Dizziness	126 (2)	714 per 1,000	950 per 1,000 (800–1,000)	RR 1.33 (1.12–1.57)	⊕⊕◯◯ LOW	Risk of bias (−1) Indirectness (−1)
	Headache	300 (1)	767 per 1,000	989 per 1,000 (905–1,000)	RR 1.29 (1.18–1.41)	⊕⊕◯◯ LOW	Risk of bias (−1) Indirectness (−1)
	Epilepsy	80 (1)	700 per 1,000	952 per 1,000 (763–1,000)	RR 1.36 (1.09–1.68)	⊕◯◯◯ VERY LOW	Risk of bias (−1) Indirectness (−1) Imprecision (−1)
	Mild TBI	80 (1)	725 per 1,000	950 per 1,000 (776–1,000)	RR 1.31 (1.07–1.61)	⊕◯◯◯ VERY LOW	Risk of bias (−1) Indirectness (−1) Imprecision (−1)
	Traumatic brain edema	40 (1)	800 per 1,000	848 per 1,000 (640–1,000)	RR 1.06 (0.80–1.41)	⊕◯◯◯ VERY LOW	Risk of bias (−1) Indirectness (−1) Imprecision (−1)
AE	Total	276 (3)	58 per 1,000	51 per 1,000 (19–133)	RR 0.88 (0.33–2.30)	⊕◯◯◯ VERY LOW	Risk of bias (−1) Imprecision (−2)
Subgroup (target symptom)	PCS	156 (1)	103 per 1,000	90 per 1,000 (34–236)	RR 0.88 (0.33–2.30)	⊕◯◯◯ VERY LOW	Risk of bias (−1) Imprecision (−2)
	Epilepsy	80 (1)	0 per 1,000	0 per 1,000 (0–0)	Not estimable	⊕⊕◯◯ LOW	Risk of bias (−1) Imprecision (−1)
	Traumatic brain edema	40 (1)	0 per 1,000	0 per 1,000 (0–0)	Not estimable	⊕⊕◯◯ LOW	Risk of bias (−1) Imprecision (−1)
Herbal medicine vs. placebo							
Fugl–Meyer assessment	Total	112 (1)	–	MD 9.63 higher (8.21–11.05 higher)	–	⊕⊕⊕◯ MODERATE	Risk of bias (−1)
Modified BI	Total	112 (1)	–	MD 18.54 higher (17.27–19.81 higher)	–	⊕⊕⊕◯ MODERATE	Risk of bias (−1)
GOS	Total (cognitive dysfunction)	53 (1)	–	MD 0 (4.17 lower−4.17 higher)	–	⊕⊕◯◯ LOW	Imprecision (−2)

(Continued)

TABLE 2 | Continued

Outcomes		No. participants (RCTs)	Anticipated absolute effects (95% CI)		Relative effect (95% CI)	Quality of evidence (GRADE)	Comments
			Risk with control group	Risk with treatment group			
QoL	Total (cognitive dysfunction)	53 (1)	–	MD 1.91 higher (9.58 lower−13.40 higher)	–	⊕⊕◯◯ LOW	Imprecision (−2)
AE	Total (cognitive dysfunction)	208 (2)	47 per 1,000	107 per 1,000 (39–295)	RR 2.29 (0.83–6.32)	⊕◯◯◯ VERY LOW	Risk of bias (−1) Inconsistency (−1) Imprecision (−2)
Herbal medicine plus conventional treatment vs. conventional treatment alone							
ADL	Total (PCS)	124 (1)	–	MD 3.30 lower (5.04–1.56 lower)	–	⊕⊕⊕◯ MODERATE	Risk of bias (−1)
BI	Total (posttraumatic hydrocephalus)	60 (1)	–	MD 11.14 higher (5.43–16.85 higher)	–	⊕⊕◯◯ LOW	Risk of bias (−1) Imprecision (−1)
SF−36 (physical component summary)	Total (PCS)	60 (1)	–	MD 3.84 higher (13.27 lower−20.95 higher)	–	⊕◯◯◯ VERY LOW	Risk of bias (−1) Imprecision (−2)
SF−36 (mental component summary)	Total (PCS)	60 (1)	–	MD 36.51 higher (13.76–59.26 higher)	–	⊕⊕◯◯ LOW	Risk of bias (−1) Imprecision (−1)
GQOLI−74 (physical health)	Total (mental disorder)	108 (1)	–	MD 11.68 higher (9.11–14.25 higher)	–	⊕⊕⊕◯ MODERATE	Risk of bias (−1)
GQOLI−74 (psychological health)	Total (mental disorder)	108 (1)	–	MD 24.41 higher (21.94–26.88 higher)	–	⊕⊕⊕◯ MODERATE	Risk of bias (−1)
GQOLI−74 (social functional status)	Total (mental disorder)	108 (1)	–	MD 13.67 higher (11.14–16.20 higher)	–	⊕⊕⊕◯ MODERATE	Risk of bias (−1)
GQOLI−74 (living condition)	Total (mental disorder)	108 (1)	–	MD 1.01 higher (1.52 lower−3.54 higher)	–	⊕⊕◯◯ LOW	Risk of bias (−1) Imprecision (−1)
TER (clinical symptom)	Total	1,429 (17)	762 per 1,000	922 per 1,000 (883–967)	RR 1.21 (1.16–1.27)	⊕⊕◯◯ LOW	Risk of bias (−1) Indirectness (−1)
Subgroup (target symptom)	PCS	652 (6)	774 per 1,000	944 per 1,000 (882–1,000)	RR 1.22 (1.14–1.30)	⊕⊕◯◯ LOW	Risk of bias (−1) Indirectness (−1)
	Mental disorder	128 (2)	781 per 1,000	938 per 1,000 (813–1,000)	RR 1.20 (1.04–1.39)	⊕◯◯◯ VERY LOW	Risk of bias (−1) Indirectness (−1) Imprecision (−1)
	Cognitive dysfunction	60 (1)	862 per 1,000	940 per 1,000 (784–1,000)	RR 1.09 (0.91–1.29)	⊕◯◯◯ VERY LOW	Risk of bias (−1) Indirectness (−1) Imprecision (−2)
	Headache	158 (2)	747 per 1,000	926 per 1,000 (799–1,000)	RR 1.24 (1.07–1.43)	⊕⊕◯◯ LOW	Risk of bias (−1) Indirectness (−1)
	Epilepsy	207 (3)	735 per 1,000	882 per 1,000 (772–1,000)	RR 1.20 (1.05–1.38)	⊕⊕◯◯ LOW	Risk of bias (−1) Indirectness (−1)
	Posttraumatic hydrocephalus	60 (1)	733 per 1,000	865 per 1,000 (667–1,000)	RR 1.18 (0.91–1.53)	⊕◯◯◯ VERY LOW	Risk of bias (−1) Indirectness (−1) Imprecision (−2)

(Continued)

TABLE 2 | Continued

Outcomes		No. participants (RCTs)	Anticipated absolute effects (95% CI)		Relative effect (95% CI)	Quality of evidence (GRADE)	Comments
			Risk with control group	Risk with treatment group			
	Mild TBI–like symptoms	164 (2)	720 per 1,000	899 per 1,000 (777–1,000)	RR 1.25 (1.08–1.46)	⊕⊕◯◯ LOW	Risk of bias (−1) Indirectness (−1)
AE	Total	1,386 (16)	78 per 1,000	50 per 1,000 (34–73)	RR 0.64 (0.44–0.93)	⊕◯◯◯ VERY LOW	Risk of bias (−1) Imprecision (−2)
Subgroup (target symptom)	PCS	592 (5)	7 per 1,000	13 per 1,000 (2–70)	RR 1.94 (0.36–10.33)	⊕◯◯◯ VERY LOW	Risk of bias (−1) Imprecision (−2)
	Mental disorder	216 (3)	178 per 1,000	96 per 1,000 (48–192)	RR 0.54 (0.27–1.08)	⊕◯◯◯ VERY LOW	Risk of bias (−1) Imprecision (−2)
	Cognitive dysfunction	130 (2)	0 per 1,000	0 per 1,000 (0–0)	Not estimable	⊕⊕◯◯ LOW	Risk of bias (−1) Imprecision (−1)
	headache	96 (1)	42 per 1,000	21 per 1,000 (2–222)	RR 0.50 (0.05–5.33)	⊕◯◯◯ VERY LOW	Risk of bias (−1) Imprecision (−2)
	Epilepsy	128 (2)	469 per 1,000	295 per 1,000 (188–473)	RR 0.63 (0.40–1.01)	⊕◯◯◯ VERY LOW	Risk of bias (−1) Inconsistency (−2) Imprecision (−2)
	Posttraumatic hydrocephalus	60 (1)	0 per 1,000	0 per 1,000 (0–0)	Not estimable	⊕⊕◯◯ LOW	Risk of bias (−1) Imprecision (−1)
	Mild TBI–like symptoms	164 (2)	0 per 1,000	0 per 1,000 (0–0)	Not estimable	⊕⊕◯◯ LOW	Risk of bias (−1) Imprecision (−1)
TESS	Total (mental disorder)	196 (3)	–	MD 1.05 lower (1.46–0.64 lower)	–	⊕⊕⊕◯ MODERATE	Risk of bias (−1)

ADL, activities of daily living; AE, adverse event; BI, Barthel index; CI, confidence interval; CT, conventional treatment; GCS, Glasgow coma scale; GOS, Glasgow outcome scale; GQOLI-74, generic quality of life inventory-74; GRADE, grading of recommendations assessment, development, and evaluation; MD, mean difference; PCS, post-concussion syndrome; QoL, quality of life; RCT, randomized controlled trial; RR, risk ratio; SF-36, 36-item short forms.

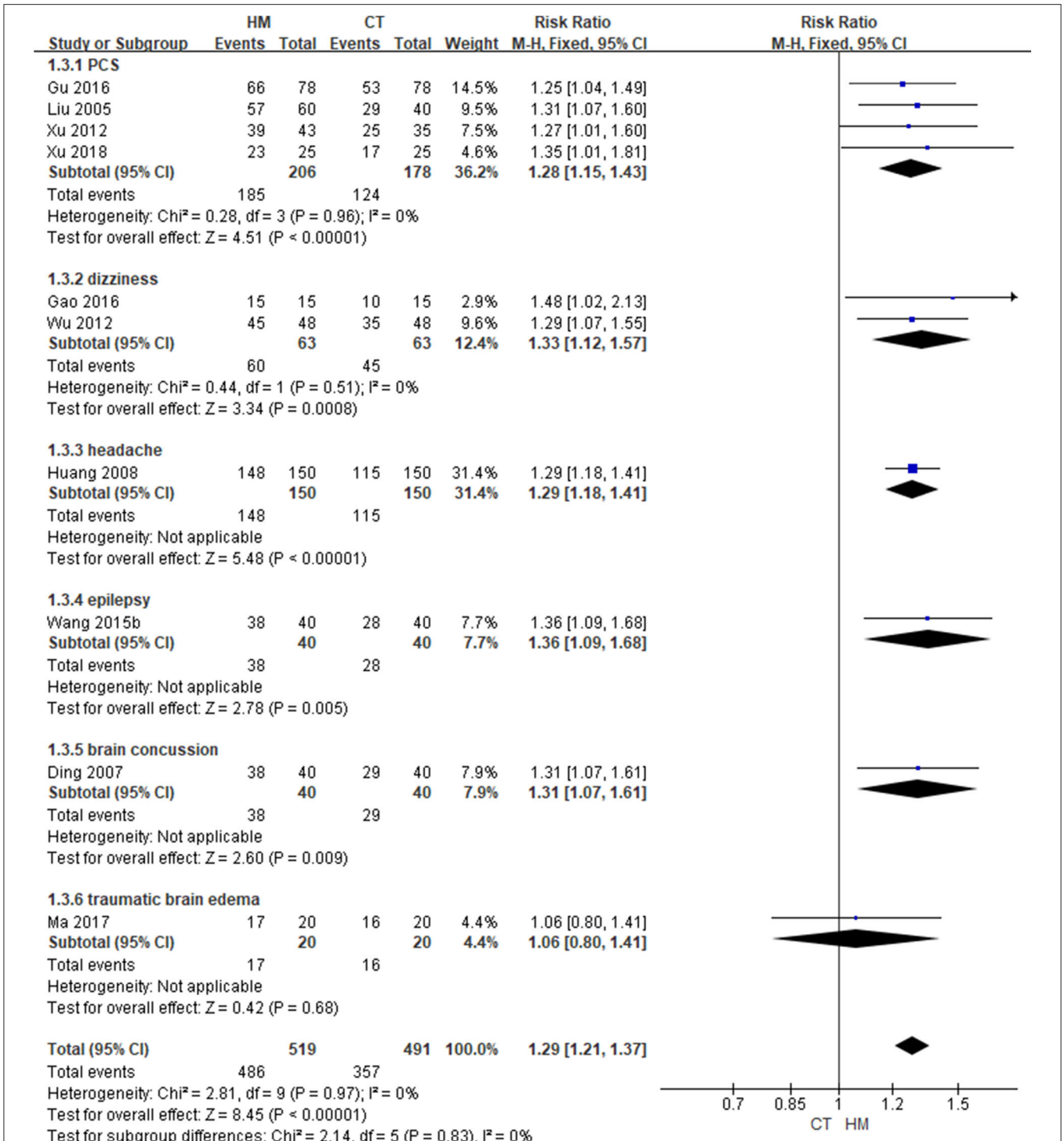

FIGURE 4 | Total Effective rate based on clinical symptoms (Comparison of herbal medicine vs. conventional treatment).

the groups after 1 month of post-intervention follow-up ($P < 0.05$) (77).

Two studies (64, 79) reported the QoL of patients after treatment. One (79) showed that patients with PCS treated using HM had significantly better mental component summary score, as measured using the SF-36 scale, than the CT alone group after 6 weeks of treatment (MD: 36.51, 95% CI: 13.76–59.26). However, there was no difference in physical component summary score (MD: 3.84, 95% CI: −13.27–20.95). Another study (64) treated patients with mental disorder for 8 weeks.

The HM group showed significantly better scores in the areas of physical health, psychological health, and social functional status domain, measured using the generic QoL inventory 74. However, there was no difference between the groups in terms of living condition (physical health: MD, 11.68, 95% CI, 9.11–14.25; psychological health: MD, 24.41, 95% CI, 21.94–26.88;

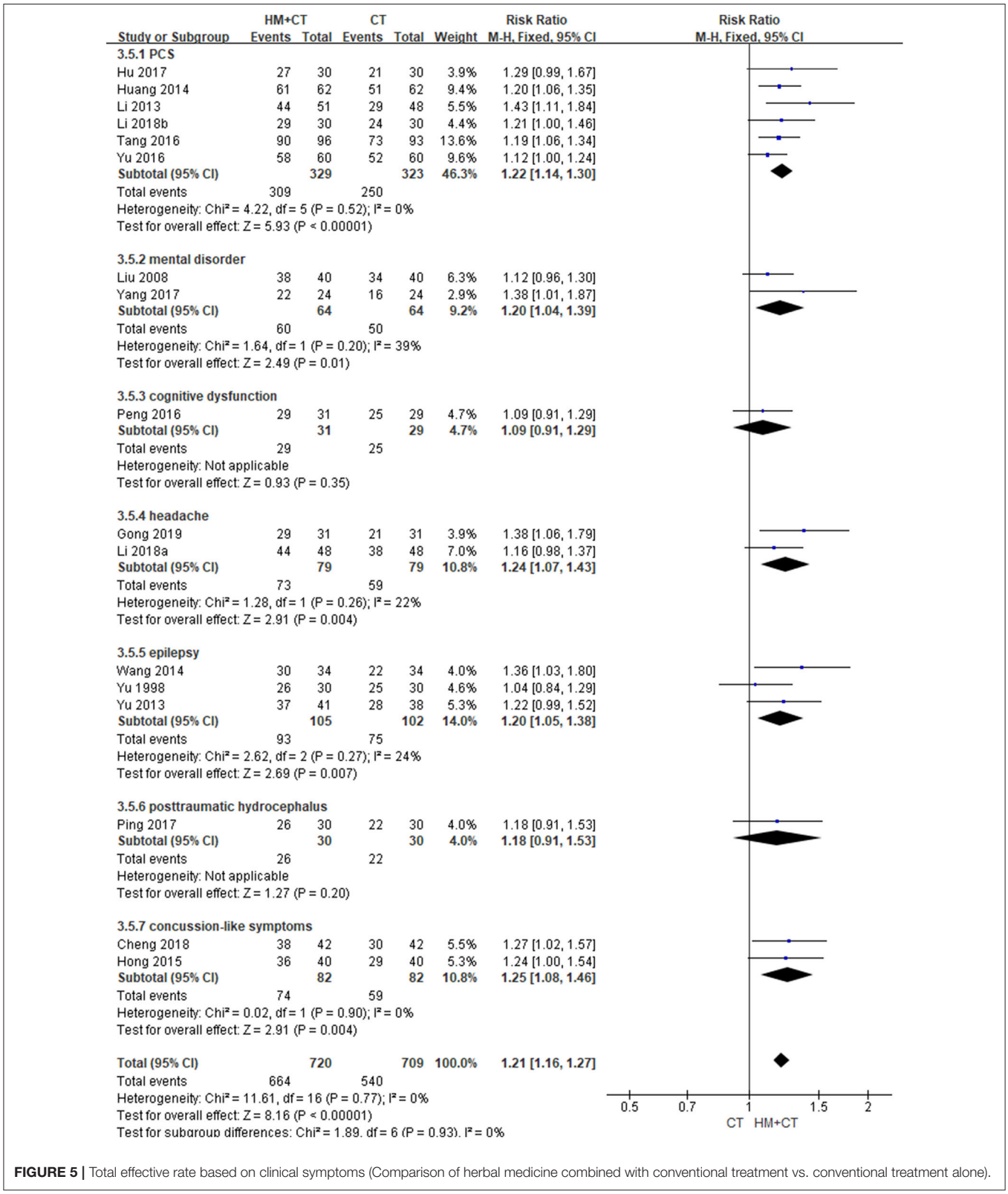

FIGURE 5 | Total effective rate based on clinical symptoms (Comparison of herbal medicine combined with conventional treatment vs. conventional treatment alone).

social functional status: MD, 13.67, 95% CI, 11.14–16.20; living condition: MD, 1.01, 95% CI,−1.52–3.54). The HM group showed significantly better TER, based on clinical symptoms (17 studies; RR: 1.21, 95% CI: 1.16–1.27, $I^2 = 0\%$) (**Figure 5**). In a subgroup analysis according to target symptoms of TBI, there were significant differences in PCS, mental disorder, headache, epilepsy, and mild TBI-like symptoms, but not in cognitive dysfunction or post-traumatic hydrocephalus (**Table 2**) (**Supplemental Digital Content 3**). However, a sensitivity analysis that excluded studies with a high risk of bias showed no difference in TER based on clinical symptoms between the two groups (**Supplemental Digital Content 4**).

When HM plus CT was administered to treat patients with PCS, neurological function, as measured using the NIHSS, was better than when CT alone was used ($P < 0.05$) (60), and cure time was significant shorter in the combination group ($P < 0.05$) (51). In patients with mental disorder after TBI, symptoms of depression (53), anxiety (53), and schizophrenia (62, 64, 66) were significantly better in the combination group than in the CT alone group ($P < 0.05$ in all cases). Furthermore, when HM plus CT was administered, cognitive function, as measured using the mini-mental state examination, was significantly improved ($P < 0.05$) (61), and the recurrence rate of headache was significantly lower than in the CT group ($P < 0.05$ in all cases) (50, 56). Two studies showed that clinical symptom relief time was significantly shorter in the combination group ($P < 0.05$ in all cases) (52, 73).

Safety

Sixteen studies (51, 55, 56, 61–64, 66, 70, 71, 73, 76, 77, 79, 80, 82) reported the incidence of AEs during the treatment period. The meta-analysis showed that the incidence of AEs was significantly lower in the HM plus CT group than in the CT alone group (RR: 0.64, 95% CI: 0.44–0.93, and $I^2 = 34\%$). Three studies (62, 64, 66) reported TESS scores after treatment in patients with mental disorder. The results showed that TESS scores were significantly lower in the combination group than in the CT group (MD: −1.05, 95% CI: −1.46 to −0.64, and $I^2 = 85\%$; **Table 2**; **Figure 6**) (**Supplemental Digital Content 3**).

Quality of Evidence

In the studies that compared HM with CT, the quality of evidence was graded as "very low" or "low" (**Table 2**). Additionally, the quality of evidence was graded as "very low" to "moderate" in studies that compared HM with a placebo, as well as in those that compared HM plus CT with CT alone (**Table 2**). The main reason for these low grades was the high risk of bias of the included RCTs. Furthermore, most findings had low precision because they did not fulfill the optimal sample size and had wide CIs. Indirect outcome measures also lowered the quality of evidence, especially in studies that measured TER as an outcome.

Publication Bias

No evidence of publication bias emerged from the funnel plots of TER based on clinical symptoms in studies that compared the effectiveness of HM with that of CT, or in studies that compared the effectiveness of HM plus CT with that of CT alone. Furthermore, the funnel plot comparing AE incidence between the HM plus CT group and the CT alone group was also symmetrical (**Figure 7**).

DISCUSSION

This review aimed to assess the effectiveness and safety of HM as a monotherapy or adjunctive therapy to conventional treatment for TBI. We conducted a comprehensive and systematic search of English, Korean, Chinese, and Japanese-language databases and retrieved a total of 37 RCTs (46–82).

In summary, when comparing HM with CT, there was no conclusive evidence in functional outcome or consciousness state in patients with traumatic brain edema because there was only one study. However, the function measured by Fugl–Meyer assessment, BI, and NIHSS was significantly improved when HM was added to CT in studies that focused on symptomatic treatment or rehabilitation. Results regarding QoL were inconsistent between the two groups after treatment. The present meta-analysis showed that the TER of various symptoms showed significantly better results in the HM group in all comparisons. However, TER is a non-validated outcome measure that is secondarily processed, and thus, assertions regarding HM's effectiveness cannot be made confidently. Regarding the safety of HM, none of the study participants showed obvious abnormalities in electrocardiogram examinations or laboratory tests, such as the blood routine, urine routine, fecal routine, and liver and kidney function tests. There was no difference in the incidence of AEs between the two groups when HM monotherapy was compared with CT or placebo. Conversely, the incidence of AEs and TESS was significantly better in the HM plus CT group than in the CT alone group. However, the risk of bias in the included studies was generally high, whereas the quality of evidence of the main findings was generally low; thus, only limited confidence can be placed in the estimate of the effect, that is, the true effect may be different from the estimate.

Interestingly, pattern identification based on blood stasis was most frequently used in the included studies. In addition, the most commonly used HM was *Xuefuzhuyu* decoction, and the commonly used single herbs comprising the HM were *Cnidii Rhizoma*, *Angelicae Gigantis Radix*, *Persicae Semen*, *Carthami Flos,* and *Paeoniae Radix Rubra*, which improve blood stasis (84, 85). In East-Asian traditional medicine, blood stasis is considered the main pathology in traumatic injury (84). According to this pathological concept, blood stasis-removing therapy is widely used to treat TBI in clinical practice, and some clinical evidence has shown that blood stasis-removing HM is effective in the treatment of TBI (86, 87). Our review does not prove that blood stasis-removing HM is effective in improving TBI, but suggests that this type of herbal medicine is promising in the field of research for TBI treatment in the future.

Many studies have tried to explain the mechanism through which HM functions in TBI, showing that HM decreases neuronal injury by increasing superoxide dismutase and catalase activities, as well as by suppressing the expression of interleukin (IL)-1, IL-6, nuclear factor kappa B, and glial fibrillary acidic protein (88). Another study showed that HM protected a

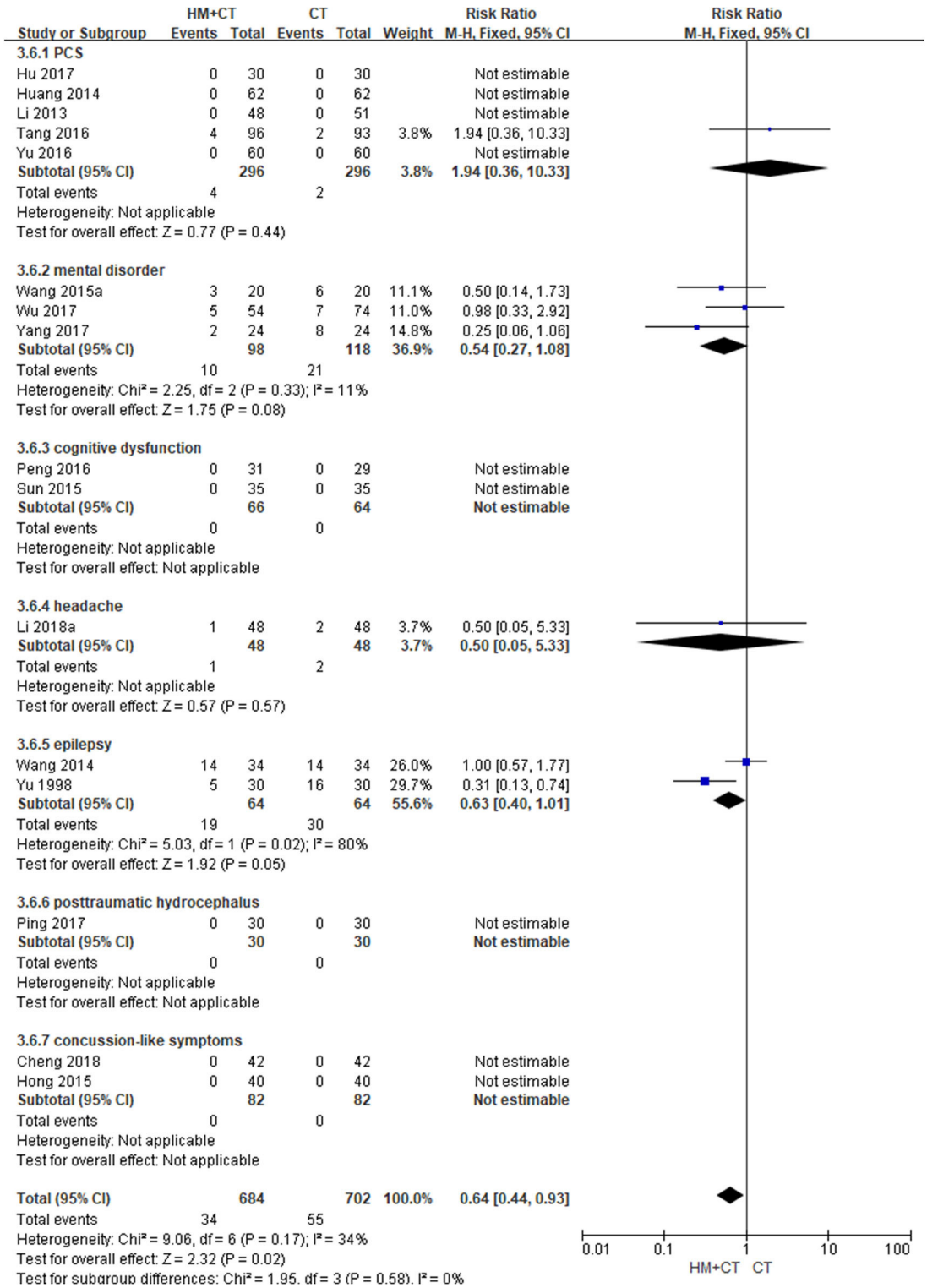

FIGURE 6 | Adverse event (Comparison of herbal medicine combined with conventional treatment vs. conventional treatment alone).

rat model of TBI, possibly via immune-promoting, anti-inflammatory, and neuroprotective effects (89). However, the underlying mechanism of HM in the treatment of TBI is still not fully understood; future studies should address this question to help establish an optimal management strategy for BI.

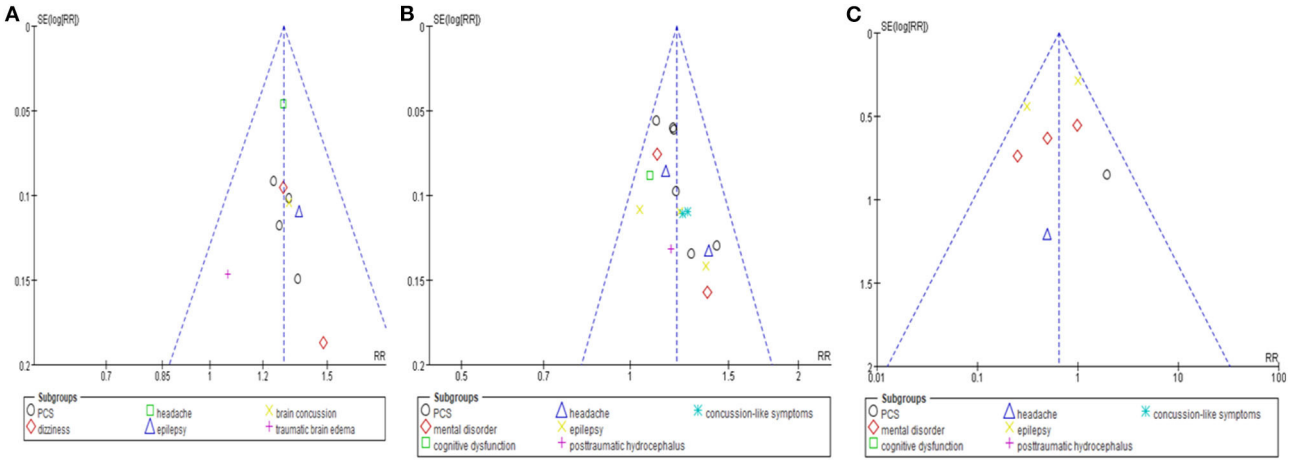

FIGURE 7 | Funnel plots of the meta-analysis. **(A)** Total effective rate based on clinical symptom. Comparison: herbal medicine vs. conventional treatment. **(B)** Total effective rate based on clinical symptom. Comparison: herbal medicine combined with conventional treatment vs. conventional treatment. **(C)** Adverse events. Comparison: herbal medicine combined with conventional treatment vs. conventional treatment.

Our review had the following limitations. Firstly, although we conducted a systematic and comprehensive search in English, Korean, Chinese, and Japanese databases, most studies were conducted and published in China. This may have resulted in reporting biases, such as language and location bias. In addition, many studies assessed TER, which is a secondarily processed outcome measure according to certain criteria, and the meta-analysis showed significant results suggesting better outcomes in the HM group. However, this non-standardized outcome measure may have caused outcome reporting bias, and the results may not have been reliable. Secondly, most of the included studies were not of high quality. In particular, many had a high risk of performance bias. Therefore, our confidence in the effect estimate, as assessed using GRADE methodology, was low. Thirdly, we attempted to perform subgroup analysis in terms of either the objective of intervention (acute management or rehabilitation) or the TBI severity, as described in the study protocol (30). However, few studies clearly specified the objective of intervention or the severity of TBI in a subgroup analysis. Finally, although we performed subgroup analysis according to different target symptoms of TBI to address heterogeneity, we could not resolve clinical heterogeneity because the participants had diverse clinical characteristics and a wide range of interventions were used in the included studies. Relatedly, because the studies showed clinical heterogeneity, we performed only a few quantitative syntheses.

The following recommendations may be considered in future studies. To evaluate the effectiveness of HM in PCS, participants should be enrolled using standardized diagnostic criteria, such as the international statistical classification of diseases and related health problems or the diagnostic and statistical manual of mental disorders. In addition, the multi-compound, multi-target nature of HM may improve a wide range of symptoms after TBI, such as PCS; therefore, the underlying molecular mechanism of HM should be studied. Particularly, priority should be given to HM and/or herb, which are especially known for ameliorating blood stasis, in further HM researches on TBI. To optimize the use of HM during treatment of TBI and to resolve the clinical heterogeneity, future studies should characterize the participants in detail, with particular focus on TBI severity and target symptoms after TBI, such as headache, mental disorder, and cognitive dysfunction, and on the objectives of HM, such as acute management or rehabilitation. In PCS, validated disease specific tools should be adopted to evaluate the effect of HM on various symptoms and deficits; these may include the Rivermead Postconcussion Symptoms Questionnaire, the World Health Organization Disability Assessment Schedule 2.0, and the British Columbia Post-concussion Symptom Inventory-Short Form (90). Finally, only three of the retrieved studies compared HM with a placebo and these showed marked clinical heterogeneity, and thus, we could not draw a definite conclusion about the efficacy of HM. Blinding of participants and personnel using placebo with the same taste, flavor, and formulation should be conducted to avoid performance bias. In future, rigorously conducted, placebo-controlled trials to evaluate the efficacy of HM in TBI should be performed considering the above implications.

CONCLUSION

The current evidence suggests that there is insufficient evidence for recommending HM for TBI in clinical practice. Although some RCTs reported that HM as an adjuvant therapy to CT may have benefits for some functional outcomes of TBI, the low quality of evidence significantly limited its reliability. Therefore, further rigorous, well-designed, high quality, placebo-controlled RCTs should be conducted to confirm these results.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are included in the article.

AUTHOR CONTRIBUTIONS

This study was conceptualized by JL. BL and C-YK performed the literature search, study selection, data extraction, and quality assessment using the risk of bias tool and GRADE approach. BL analyzed the data and C-YK critically double-checked the data analysis. BL and C-YK drafted the manuscript. All authors interpreted the data and critically reviewed the manuscript. The draft was reviewed and edited by HK, JL, and H-GJ. Resources were provided by JL. This study was supervised by HK and H-GJ. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.00772/full#supplementary-material

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PERSPECTIVE
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Perspective: Cognitive Behavioral Therapy for Insomnia Is a Promising Intervention for Mild Traumatic Brain Injury

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Mild traumatic brain injury (mTBI) is a significant public health problem. Insomnia is one of the most common symptoms of TBI, occurring in 30–50% of patients with TBI, and is more frequently reported in patients with mild as opposed to moderate or severe TBI. Although insomnia may be precipitated by mTBI, it is unlikely to subside on its own without specific treatment even after symptoms of mTBI reduce or remit. Insomnia is a novel, highly modifiable treatment target in mTBI, treatment of which has the potential to make broad positive impacts on the symptoms and recovery following brain injury. Cognitive-behavioral therapy for insomnia (CBT-I) is the front-line intervention for insomnia and has demonstrated effectiveness across clinical trials; between 70 and 80% of patients with insomnia experience enduring benefit from CBT-I and about 50% experience clinical remission. Examining an existing model of the development of insomnia in the context of mTBI suggests CBT-I may be effective for insomnia initiated or exacerbated by sustaining a mTBI, but this hypothesis has yet to be tested via clinical trial. Thus, more research supporting the use of CBT-I in special populations such as mTBI is warranted. The current paper provides a background on existing evidence for using CBT-I in the context of TBI, raises key challenges, and suggests considerations for future directions including need for increased screening and assessment of sleep disorders in the context of TBI, examining efficacy of CBT-I in TBI, and exploring factors that impact dissemination and delivery of CBT-I in TBI.

Keywords: CBT (cognitive-behavioral therapy), insomnia, traumatic brain injury, mild traumatic brain injury (mTBI), sleep, cognitive behavioral therapy for insomnia (CBT-I)

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BACKGROUND

Mild traumatic brain injury (mTBI) is a significant public health problem for many populations including children, adolescents, young adults, older adults, athletes, and military personnel. mTBI is associated with numerous negative sequalae including mental and physical health, work and social functioning, and financial burden to the injured individual and society. Up to 80% of the 1.7 million traumatic brain injuries (TBI) in America each year are classified as mild (1). Despite the prevalence of mTBI, no compelling treatments exist. More work exploring treatment targets in mTBI is a high

priority. mTBI commonly precipitates or exacerbates sleep disturbances which can in turn worsen other symptoms or sequelae of mTBI (e.g., fatigue, depression, pain) and interfere with both neural and physical rehabilitation (2). Insomnia, the most common sleep disturbance following mTBI, is a largely unexplored and highly modifiable intervention target for improving rehabilitation and long-term outcomes in mTBI and thus an important area of focus for future research.

INSOMNIA IN TBI

Untreated insomnia is associated with numerous mental and physical health sequelae (3), increased healthcare utilization (4), work problems (5), and financial burden on both the individual and society (6). Insomnia is one of the most common symptoms of TBI; 30–50% of patients report insomnia that developed or worsened following TBI (7) which is substantially elevated above general population prevalence [i.e., 6–10%; (8, 9)]. Insomnia can develop immediately following the TBI or later during the recovery period and is unlikely to remit without targeted treatment even after TBI symptoms reduce or remit (10). Insomnia disorder is characterized by disturbances in both sleep (i.e., difficulty initiating or maintaining sleep) and wake (e.g., fatigue, difficulty concentrating) domains which, paired together, cause clinically significant distress or impairment (11). Counter to expectations, insomnia is reported at a higher rate in individuals with mTBI compared to moderate or severe TBI (12–15). Reasons for this apparent paradox are not entirely clear, but individuals with mTBI may be more likely to notice or report sleep disturbances compared to individuals with more severe TBI who are coping with greater impairments in memory and cognitive functioning and may have decreased self-awareness (15). Many symptoms of mTBI and insomnia overlap, including fatigue, poor attention and concentration, irritability and poor mood, and memory difficulties. Although insomnia has traditionally been conceptualized as a symptom secondary to a primary disorder (e.g., depression), recent thinking suggests insomnia takes on a life of its own and warrants separate, targeted clinical focus (16). Because of the global impact of insomnia, successful treatment has the potential to substantially improve symptoms and recovery following mTBI.

COGNITIVE-BEHAVIORAL THERAPY FOR INSOMNIA

Cognitive-behavioral therapy for insomnia (CBT-I) is efficacious across many populations (17–23) and thus is the first-line treatment for insomnia as recognized by multiple organizations including the American College of Physicians (24), European Sleep Research Society (25), American Academy of Sleep Medicine (26), NIH Consensus State-of-the-Science Conference (27), and VA/DoD clinical practice guidelines (28). Numerous clinical trials and systematic reviews demonstrated between 70 and 80% of patients with insomnia experience enduring benefit from CBT-I and about 50% experience clinical remission (29). CBT-I typically includes multiple cognitive and behavioral treatment components, most commonly sleep restriction, stimulus control, relaxation training, cognitive therapy, and psychoeducation/sleep hygiene (29). Taken together, CBT-I reduces wakefulness and promotes sleep in the bed and bedroom, regularizes the sleep/wake cycle, reduces sleep-related anxiety and arousal, and reshapes unhelpful thoughts about sleep. A typical course of CBT-I is delivered to a patient across 4–8 weekly sessions although many alternative formats and delivery strategies have demonstrated efficacy (e.g., internet-delivered, group therapy, abbreviated treatment). Evidence suggests in addition to clinical psychologists and psychiatrists, nurse practitioners (30) and masters-level clinicians (31) can be trained to deliver CBT-I effectively making it a versatile, flexible intervention. However, uptake of CBT-I is low due to numerous barriers including limited patient and provider awareness of the intervention and lack of trained providers (32). It is difficult to ascertain the exact number of individuals competently trained to delivery CBT-I, although existing data suggests a paucity of trained providers. There are currently only 106 providers certified in the provision of Behavioral Sleep Medicine [which encompasses CBT-I; (33)]. Additionally, per a 2016 report 598 mental health providers completed training in CBT-I via the Veterans' Administration rollout (34). It is likely that many more providers (e.g., psychologists) deliver CBT-I, but the amount and quality of services provided is unknown. Judging from anecdotal reports of high referral volume and extensive waitlists within clinics providing behavioral sleep medicine services, it is likely that the current demand for CBT-I exceeds capacity. Thus, providers embedded in settings with a high volume of patients with mTBI may consider seeking training in CBT-I (e.g., www.cbtiweb.org) to help alleviate this demand.

PHARMACOLOGICAL TREATMENT OF INSOMNIA IN MTBI

CBT-I is typically preferred over pharmacological interventions for insomnia (i.e., hypnotics) because in comparison it has consistent long-term benefit and a minimal side effect profile [for a review of pharmacological interventions for insomnia in TBI, see (35)]. Side effects of common hypnotics (e.g., benzodiazepines, non-benzodiazepine receptor agonists) are particularly concerning for patients with mTBI given overlap with symptoms of mTBI including daytime sleepiness, fatigue, dizziness, headache, mental slowing, and attentional and memory difficulties. Further, emerging evidence suggests long-term use of hypnotics may be associated with increased risk of dementia (36–38), particularly for TBI patients (39), and mortality (40, 41). Given the potential risks of hypnotics, CBT-I is a clear first choice for treatment of insomnia in patients with mTBI, although they need not be mutually exclusive treatments and can in some circumstances be used together effectively.

A CONCEPTUAL MODEL OF THE DEVELOPMENT OF INSOMNIA IN THE CONTEXT OF MTBI

The behavioral or "3P" model of insomnia posits three primary factors which contribute to the development and maintenance

of chronic insomnia over time: predisposing, precipitating, and perpetuating factors (42). The 3P model provides some insight as to how CBT-I could be particularly beneficial to an individual with co-occurring mTBI and insomnia. The first factor of the model, termed "predisposing," includes elements which inherently put an individual at increased risk for insomnia (e.g., genetics, personality, chronotype). For a given individual with mTBI, prior history of insomnia and prior head injuries are likely elements which could contribute to a predisposition to insomnia. Although predisposition may increase risk for insomnia, it generally does not produce insomnia at the clinical level in the absence of the other two factors (42). The second factor, "precipitating event," suggests in most cases an identifiable event or set of events (e.g., military service, illness, sports injury) contributes to the development of insomnia in the acute phase (i.e., <1 month). Certainly, a mTBI could serve as the event precipitating a bout of insomnia; many acute changes following mTBI such as physical and/or neurological changes, pain, psychological difficulties (e.g., depression, posttraumatic stress), and changes in activity level are commonly associated with the development of insomnia (10). However, insomnia is unlikely to persist into the recovery period (e.g., after mTBI-related changes subside or adjustment to stressors occurs) in the absence of the third factor (42).

The third factor of the behavioral model of insomnia, "perpetuating," refers to the tendency of individuals with insomnia in the acute phase to make changes to their behaviors and thoughts related to sleep in order to attempt to cope with the development of sleep disturbances (42). For example, a patient with mTBI who subsequently developed insomnia might attempt to go to bed early, sleep in late, or take daytime naps to "make up for" sleep lost due to insomnia. During the acute phase following mTBI, patients can experience a period of increased sleep need (i.e., pleiosomnia) above the pre-injury baseline (43), which may be followed by an eventual return to baseline sleep duration, although this phenomenon may be more common or severe among individuals with pain (44) or with more severe injuries (45). However, the patient may continue to attempt to sleep longer (i.e., spend more time in bed) even after they are no longer able to produce sleep at the increased quantity. This will result in extended periods of wakefulness in the bedroom environment, which decreases the association between the bedroom and sleep and thus weakens the sleep drive. Behavioral changes like extending wakefulness in bed, in contrast to what the individual intends, can perpetuate insomnia into the chronic phase. Similarly, the patient might start having thoughts related to their sleep and recent injury such as "Lack of sleep will negatively impact my recovery," or "My injury permanently damaged my ability to sleep well" which in turn increase hyperarousal, rumination and worry. This makes achieving restful sleep even more difficult. This collection of perpetuating behaviors and thoughts thus becomes the target on which CBT-I intervenes (46). For an individual with a mTBI who may not receive much assistance with sleep following the injury (aside recommendations to "get enough sleep,") CBT-I could be a crucial intervention which changes the course of insomnia and thus recovery from mTBI. Although it is beyond the scope of this article to recommend specific adaptations of CBT-I in the context of mTBI, Ouellet et al. (47) recently published a book which comprehensively addresses this topic for providers.

POTENTIAL IMPACTS OF CBT-I ON MTBI

One domain in which CBT-I could be particularly impactful for mTBI patients is cognitive functioning. A recent meta-analysis (48) demonstrated insomnia is associated with small to moderate deficits in cognitive functioning across numerous domains, including subjective cognitive performance and objective assessments of perceptual functioning, working memory (i.e., retention/capacity), episodic memory, complex attention, alertness, and executive functioning (i.e., problem solving). Each of these impairments could impact a patient's ability to engage in mTBI rehabilitation activities (e.g., following directions, completing out-of-session tasks, attending appointments) and thus reducing the burden of insomnia on cognitive functioning could improve engagement (49). Some existing evidence in populations without TBI suggests treating insomnia via CBT-I or other behavioral interventions may improve subjectively-assessed cognitive functioning (50), and preliminary evidence suggests some effect for objectively-assessed cognitive functioning (51, 52). However, more work is needed to understand the potential for improving cognitive functioning for patients with mTBI via CBT-I.

Another domain in which insomnia treatment could impact symptoms and rehabilitation course in mTBI is the improvement of psychosocial functioning (2). Unlike other forms of cognitive-behavioral therapy, CBT-I has a specific focus on sleep problems and in a manualized form does not directly treat other mental health symptoms. Nevertheless, a growing literature suggests treating insomnia in clinical populations is associated with significant improvements in pain (53), depression (54), anxiety (55), and posttraumatic stress symptoms (56). Each of these problems co-occurring with mTBI could markedly interfere with engagement in rehabilitation efforts or delay recovery (2). However, psychological treatment for each of these problems typically takes longer and may be less appealing to patients due to stigma or avoidance compared to CBT-I. Insomnia may be a more "approachable" treatment target than other psychosocial problems as it specifically targets sleep problems which may be less stigmatized than depression, anxiety, pain, and posttraumatic stress, yet CBT-I can also result in improvement across these domains in a relatively short time frame (57).

EXISTING AND ONGOING RESEARCH ON TREATMENT OF INSOMNIA AND SLEEP PROBLEMS IN MTBI

Research on CBT-I in Mild to Severe TBI

Despite the promise of CBT-I for patients with mTBI, previous work is limited (58). CBT-I has not been sufficiently examined in this population and there are no targeted treatments for TBI-related insomnia (59). In 2007, Ouellet et al. conducted a single-case experimental study of 8 weeks of CBT-I for 11

patients with mild to severe TBI and insomnia which developed following the TBI. The results demonstrated a significant improvement in sleep efficiency (i.e., the percentage of time in bed spent asleep) from pretreatment to posttreatment and 3-month follow-up (60). This study provides some preliminary evidence CBT-I may be useful in this population but is limited by small sample size and inclusion of a range of TBI severities.

Research on Non-CBT-I Sleep Interventions in Mild to Severe TBI

Although studies of CBT-I for patients with TBI are scarce, some studies of treating related sleep problems in TBI can provide additional preliminary support. One study randomly assigned 24 adults with mild to severe TBI and complaints of sleep problems or fatigue to either treatment as usual or an 8-session cognitive-behavioral therapy (CBT) intervention for sleep and fatigue that included some elements of CBT-I. The results suggested the participants who received the CBT intervention demonstrated improvements in sleep quality, fatigue, and depression (61). Similarly, a pilot randomized controlled trial randomly assigned 24 adults with mild to moderate TBI and sleep difficulties to either a psychoeducation control condition or a 6-sesssion online CBT intervention that included some elements of CBT-I. Results suggested participants in the intervention condition experienced improvements in sleep disturbances but there was no effect for cognitive functioning or post-concussive symptoms (62). Finally, a prospective longitudinal study delivered individualized sleep recommendations (including sleep hygiene, medication, or sleep apnea treatment) to twelve adults with mild to severe TBI and found significant improvements in insomnia severity, depression severity, language, and speed of language processing (63). These studies provide some support for the impact of improving sleep in mild TBI but share significant limitations including heterogeneous TBI severity and sleep complaints, use of broad interventions, and small sample sizes. Further work examining targeted treatment (i.e., CBT-I) for diagnosed insomnia for individuals with mTBI will enhance understanding of the potential effect of CBT-I on the pervasive symptoms of mTBI.

Ongoing Research on CBT-I in mTBI

Our group is currently conducting the first large randomized controlled trial of CBT-I in patients with mTBI (ClinicalTrials.gov identifier: NCT03261674). In this study, we will randomize 120 Veterans with mTBI to receiving either CBT-I or another behavioral treatment for insomnia and examine the effect of treatment on insomnia symptoms, quality of life, and functional outcomes. We will also explore potential mediators and moderators of response including cognitive arousal, initial sleep characteristics, posttraumatic stress symptoms, pain, chronic stress and inflammation. This study will help to fill the substantial gap in the literature of insomnia treatment in patients with mTBI.

CURRENT CHALLENGES IN USING CBT-I IN MTBI

More work examining the use of CBT-I in patients with mTBI is needed because they may present unique challenges for insomnia treatment compared to other populations. We have summarized some of the potential challenges here:

1. **Patients with mTBI struggle with attention, concentration, mental fatigue, or forgetfulness which may complicate the implementation of behavioral changes at home.** CBT-I relies on the patient to adhere to a set schedule, make changes to their bedtime routine, and complete daily sleep logs. Poor adherence to these guidelines may reduce the efficacy of the intervention or require some adaptation of CBT-I materials (e.g., additional reminders, simplified handouts, provisions for coping with fatigue, enlistment of a family member for support), although to date research has not been conducted to explore the efficacy of such modifications. However, one recent study of CBT-I for patients with mild cognitive impairment (64) resulted in significant improvements in sleep and cognitive functioning, suggesting CBT-I can be efficacious for individuals with memory impairment.
2. **Insomnia may co-occur with other sleep disturbances or disorders for which CBT-I may not be effective.** For example, mTBI is frequently associated with increased rates of daytime fatigue, obstructive sleep apnea, sleep fragmentation, or circadian rhythm disturbances (65). Although CBT-I can be safely used for insomnia in the context of these co-occurring sleep-wake disturbances, the benefit of CBT-I may be attenuated because symptoms from these disorders are unlikely to remit without targeted treatment. In other words, even if the insomnia is successfully treated, other untreated sleep disturbances may remain and cause continued impairment. In particular, patients with mTBI are more likely to experience daytime sleepiness (66) and severe fatigue (67). Daytime sleepiness is uncommon in a "typical" course of insomnia (i.e., people with insomnia without mTBI) and is not directly addressed by CBT-I. Although fatigue is common in insomnia, mTBI-related fatigue may not be fully alleviated by the typical strategies of CBT-I and thus require additional management. Both daytime sleepiness and fatigue may require the addition of alertness-promoting strategies to increase safety and maximize functioning and adherence to CBT-I. Similarly, the presence of circadian rhythm disturbances in mTBI may require specific attention that would typically be outside the scope of CBT-I but which could be successfully addressed by the addition of treatment components [e.g., light therapy; (68)].
3. **Individuals with mTBI who return to their typical activities are at increased risk for sustaining another head injury which may reduce the appropriateness of CBT-I.** In sustaining another head injury, the course of CBT-I may be disrupted or set back. For example, during the acute phase of injury, which can be associated with increased need for sleep rather than insomnia, the recommendations of CBT-I are unlikely to be beneficial and may require a delay in

insomnia treatment. Little is known about the optimal time course of delivering CBT-I in the context of mTBI.

Given these considerations, more work is needed to understand the potential benefit of both unmodified and adapted CBT-I for patients with mTBI.

FUTURE DIRECTIONS

There is a clear need for further research examining the potential utility of treating insomnia to improve the symptoms, recovery and rehabilitation of individuals with mTBI. Below, we have outlined priorities for future research in this area.

1. **Improvement of screening and assessment of sleep disorders in both clinical and research settings within the context of mTBI at all phases of injury using a validated and comprehensive measurement strategy [i.e., both subjective and objective sleep assessment; (59)] is vital.** Screening for sleep disorders should be standard practice for individuals with mTBI. This will contribute to a better understanding of the nature and course of sleep disturbances in mTBI and inform treatment development. Similarly, it is crucial to increase the integration of sleep services within the mTBI treatment context and develop the evidence base for targeted sleep-related recommendations and interventions. Detailed information about how to conduct a thorough assessment for sleep disorders among individuals with mTBI is presented in a recent book by Ouellet et al. (47).
2. **More clinical trials are needed which examine the impact of cognitive-behavioral interventions for sleep disturbances in mTBI.** As discussed above, our group has an ongoing trial to contribute to the evidence based for CBT-I among military veterans with mTBI. Additional studies are required to establish the efficacy and effectiveness of CBT-I or related interventions among additional subpopulations including children and adolescents, emerging adults, older adults, athletes, and mTBI comorbid to psychiatric or medical issues. We might expect that the variable presentation of mTBI among these subgroups could impact the delivery of CBT-I. For example, athletes may be focused on return to play or maximizing performance, which could change the focus of cognitive therapy. Alternatively, individuals with comorbid mental health diagnoses may benefit from the addition of cognitive-behavioral interventions which are not necessarily standard in CBT-I, such as behavioral activation. Once the efficacy of well-tested interventions (e.g., CBT-I) for specific sleep disorders is established in the mTBI population, adaptations that meet the specific needs of individuals with mTBI, and specific subgroups within the mTBI umbrella, will likely maximize benefits. For additional recommendations regarding specific directions for research on sleep and CBT-I in the context of mTBI, see a research agenda proposed by Wickwire et al. (68) and a recent review by Ford et al. (69).
3. **In addition to adaptations of CBT-I, the efficacy of other cognitive-behavioral interventions for sleep disorders should be explored for individuals with mTBI.** Transdiagnostic sleep interventions [e.g., TranS-C; (70)] that address sleep health may be useful to treat broad and co-occurring sleep disturbances. Although transdiagnostic sleep interventions have a smaller evidence base than CBT-I, they are an important direction of future research given the constellation of overlapping sleep complaints in mTBI. Similarly, acceptance-based nonpharmacological treatment of insomnia [e.g., mindfulness-based therapy for insomnia; (71)] might fit particularly well with the presentation of patients with CBT-I who may be coping with substantial and enduring changes to health and functioning. However, these interventions have yet to be tested among individuals with mTBI.
4. **Additional research is needed to understand the optimal timing of CBT-I delivery, potential modifications or adaptations, and which subpopulations are most likely to benefit.** Little is known about the ideal timing of the delivery of CBT-I to improve outcomes in mTBI. For example, should CBT-I be initiated immediately following the acute phase, or later on in recovery? What "dose" of CBT-I is optimal (e.g., typical dose of 6–8 sessions, or is a longer course required?) Can prevention strategies be put in to place early in the post-injury phase to minimize the development of poor sleep outcomes? As mentioned previously, adaptations may be needed to optimize CBT-I for TBI populations. Relatedly, little is known about which subpopulations of TBI patients (e.g., severity, co-occurring conditions) can be appropriately and effectively treated with CBT-I. This is a distal future direction, if initial work exploring the efficacy of CBT-I in the context of TBI demonstrates a promising signal.
5. **Further research is needed to explore the immediate and long-term effects of TBI sustained in childhood and young adulthood.** Most existing treatment research has focused on TBI sustained in adulthood, despite compelling evidence that sustaining TBI in childhood can increase the risk of sleep disturbances as long as 20 years after the event (72).
6. **It is important to consider the potential broader impact of CBT-I in the context of mTBI, particularly with regard to co-occurring conditions.** Nonpharmacological sleep interventions like CBT-I may indirectly improve symptoms of common co-occurring psychosocial problems (e.g., depression, chronic pain, posttraumatic stress) in mTBI. Another important area of exploration is how access to CBT-I might be linked to more distal outcomes including return to work or other meaningful activities, social engagement, and health-related quality of life. Along these lines, it is important to explore how the effective treatment of insomnia might change the course or evolution of cognitive and other mTBI-related symptoms.
7. **More research is needed to explore barriers to dissemination and implementation of sleep-focused interventions at the systemic level and design targeted programs to address these barriers.** It is especially important to consider leveraging existing resources to increase the dissemination of CBT-I (e.g., telehealth, online-delivered interventions) in populations with individualized needs.

Increased access to training in CBT-I and sleep assessment, as well as availability of assessment and treatment tools are both crucial strategies that can help overcome the current provider-level barriers which limit access for individuals with CBT-I.

CONCLUSIONS

In sum, sleep disturbances like insomnia are a novel, modifiable treatment target which may have a broad positive impact on the symptoms and recovery following mTBI. Thus, treating insomnia in individuals with mTBI could be a priority for clinicians and researchers alike. In this context, behavioral sleep medicine interventions like CBT-I should be seriously considered as they typically do not add to medication burden, have a strong existing evidence base, and may be more acceptable to patients than medication (57). CBT-I is well-accepted as an effective treatment for insomnia in many populations and co-occurring conditions, but it has not yet been adequately tested in the context of TBI and specific challenges remain. Thus, more research is needed to address screening and assessment procedures of sleep problems in TBI, efficacy, limitations, and benefits of CBT-I in TBI, and individualized factors which may affect the delivery and utility of CBT-I in TBI.

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BRIEF RESEARCH REPORT
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Cerebral Perfusion Effects of Cognitive Training and Transcranial Direct Current Stimulation in Mild-Moderate TBI

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Background: Persistent post-traumatic symptoms (PPS) after traumatic brain injury (TBI) can lead to significant chronic functional impairment. Pseudocontinuous arterial spin labeling (pCASL) has been used in multiple studies to explore changes in cerebral blood flow (CBF) that may result in acute and chronic TBI, and is a promising neuroimaging modality for assessing response to therapies.

Methods: Twenty-four subjects with chronic mild-moderate TBI (mmTBI) were enrolled in a pilot study of 10 days of computerized executive function training combined with active or sham anodal transcranial direct current stimulation (tDCS) for treatment of cognitive PPS. Behavioral surveys, neuropsychological testing, and magnetic resonance imaging (MRI) with pCASL sequences to assess global and regional CBF were obtained before and after the training protocol.

Results: Robust improvements in depression, anxiety, complex attention, and executive function were seen in both active and sham groups between the baseline and post-treatment visits. Global CBF decreased over time, with differences in regional CBF noted in the right inferior frontal gyrus (IFG). Active stimulation was associated with static or increased CBF in the right IFG, whereas sham was associated with reduced CBF. Neuropsychological performance and behavioral symptoms were not associated with changes in CBF.

Discussion: The current study suggests a complex picture between mmTBI, cerebral perfusion, and recovery. Changes in CBF may result from physiologic effect of the intervention, compensatory neural mechanisms, or confounding factors. Limitations include a small sample size and heterogenous injury sample, but these findings suggest promising directions for future studies of cognitive training paradigms in mmTBI.

Keywords: traumatic brain injury, cognitive training, cerebral perfusion, pCASL, transcranial direct current stimulation

BACKGROUND

A significant minority of patients with mild traumatic brain injury (mTBI), up to 33%, go on to experience functional impairment a year later (1, 2). These persistent post-traumatic symptoms (PPS) can range from the somatic (dizziness, headaches, light sensitivity) to the cognitive (difficulty focusing, impaired memory) and emotional realms (depression, irritability, anxiety) (3). Historically these were thought to be due to poor coping with stress, or malingering (4, 5). However, advanced imaging has contributed significantly to our current understanding of the acute and chronic sequelae of mTBI, and expanded the possible etiologies of PPS to not only include psychological phenomena but also neurological factors (6–10).

Candidate mechanisms of PPS include microscopic axonal shearing and microhemorrhage (11); functional connectivity abnormalities (7); and ongoing neuroinflammation (12). One of the more promising hypotheses receiving significant scientific attention is that of abnormal cerebral perfusion, in which traumatic injury causes impaired neurovascular coupling and mismatch between neuronal metabolic demand and cerebral blood flow (CBF) (13–16). There is ample grounding for this pathophysiology in animal models and in severe TBI in humans (17–19), however, noninvasively detecting changes in CBF in more mild injuries has proved more challenging (20). Arterial spin-labeling (ASL) and pseudocontinuous ASL (pCASL) magnetic resonance imaging sequences are techniques for measuring cerebral perfusion that have gained traction recently. It has permitted quantification of both global and regional CBF without use of injected or inhaled agents (21, 22), based on the premise of magnetically labeling arterial blood protons prior to their flowing into a region of interest to act as an endogenous "tracer." (21)

Data from multiple studies suggest that mTBI results in a state of abnormal cerebral perfusion compared to healthy controls. The most frequent finding in studies utilizing ASL/pCASL across multiple age ranges (pediatric vs. adult), injury severities (mild/moderate/severe), timeframes (acute vs. chronic), and injury contexts is that of decreased perfusion. Wang et al. in 2016 demonstrated frontotemporal decreased CBF following subacute sport-related concussion in 18 young adult football players compared to 19 age-matched nonconcussed controls (23), as well as in a pediatric sample in 2015 (24). Clark et al. determined that reduced CBF was associated with decreased white matter integrity in 37 Veterans with chronic mild-moderate TBI (25). As severity of injury increases from mild to moderate and severe, there is a greater likelihood of decreased perfusion being present (26, 27). Newsome et al. found reduced CBF in right non-prefrontal regions in seven adolescents with chronic moderate-severe TBI, while Kim et al. in 2010 examined 27 chronic moderate-severe TBI patients and 22 matched controls with ASL and found globally decreased CBF in the TBI group, along with regional CBF reductions in posterior cingulate, thalamic, and frontal areas (26). This group also used resting and task-based ASL sequences to detect occipital and temporal hypoperfusion in 2012 in a cohort of 21 moderate-severe TBI patients (27). CBF remains abnormal into the chronic phase (28–32), and has been associated cognitive performance (27), symptom severity (32) and recovery (30).

However, several studies have found increased CBF in acute mTBI, especially in symptomatic cases. For instance, Doshi et al. in 2015 using ASL after acute mTBI found that in 14 patients with acute mTBI regional CBF was increased compared to 18 healthy controls (33). Similarly, Stephens et al. in 2018 found CBF increased in the left dorsal cingulate gyrus and left insula in 15 teenage athletes with subacute sport-related concussion compared with 15 age-matched controls (34). Finally, Barlow found that CBF was higher than controls in patients with symptomatic pediatric concussion, but lower than controls in asymptomatic pediatric concussion (30). It is apparent that a consistent pattern of change in CBF due to injury or recovery as measured by pCASL still needs to be established.

Cerebral perfusion and neurovascular coupling changes related to treatment may also be evaluated using pCASL sequences. Transcranial direct current stimulation (tDCS) (35), a type of noninvasive neuromodulation, has been shown in both animal and human studies to modulate CBF, depending on the parameters of the stimulation (excitatory vs. inhibitory, respectively) (36–40). Its promise as a treatment for cognitive deficits in mmTBI has been observed in multiple studies (41–44), and in a single small trial changes in perfusion tomography were seen following tDCS in a moderate-severe TBI population (45). However, no studies have measured cerebral perfusion with pCASL as a correlate of improvement with training or tDCS in mild or moderate injury (mmTBI). Therefore, this study aims to identify whether anodal tDCS applied to the left dorsolateral prefrontal cortex paired with a cognitive training protocol in mmTBI patients results in changes in CBF on pCASL sequences. It is hypothesized that anodal tDCS will result in regional perfusion increases, as well as improvements in cognitive performance and symptoms, compared to sham tDCS.

MATERIALS AND METHODS

Subjects with either mild or moderate TBI within the past 15 years were recruited via local brain injury clinics, brain injury advocacy centers, community flyers, and medical record search. Forty subjects aged 18–59 who had experienced mild or moderate TBI between 3 months and 15 years prior to study entry with persistent cognitive symptoms were screened and enrolled in the study. Subjects were randomized to receive either active or sham tDCS paired with cognitive training to improve executive functions and mood. Each patient underwent pre- and post-intervention testing, which included demographic assessment and medical history, TBI severity assessment, screening for contraindications to tDCS, postconcussive and behavioral symptom assessment, and neuropsychological testing. Of the forty subjects enrolled, a subset of twenty-four completed baseline and post-treatment magnetic resonance imaging scans, including pCASL. The UNM Health Science Center Institutional Review Board reviewed and approved this study.

Inclusion Criteria

Subjects qualified for enrollment in the study if they met the following inclusion criteria: (1) age 18–59; (2) have suffered a mild or moderate TBI ["mild" defined as having had loss of consciousness (LOC) <30 min, received a Glasgow coma scale (GCS) score of between 13 and 15 upon ED evaluation (if available), and experienced <24 h of post-traumatic amnesia (PTA); moderate defined as LOC between 30 min and 24 h, GCS between 9 and 12, and PTA between 24 h and 7 days]; (3) were injured between 3 months and 15 years ago; (4) report at least 1 out of 4 cognitive symptoms on the Neurobehavioral Symptom Inventory (NSI). Potential participants were excluded from participation in this study for: (1) a history of other neurological disease, seizures, or psychosis; (2) history of recent (within 2 years) substance/alcohol dependence; (3) any discontinuity in skull electrical conductivity; (4) any implanted electrical device (e.g., pacemaker); (5) medical admission or hospital visit within the last 3 weeks; (6) change in any psychotropic medications in the previous 2 months; (7) inability to complete the protocol; (8) appointment of a legal representative, as assessed via direct inquiry of the subject or a designated trusted other; (10) inability to provide informed consent; (11) pregnancy, current incarceration, or limited English proficiency.

Demographic Data

Basic demographic data regarding the subject were recorded, including age, sex, years of education, handedness, use of common stimulants such as caffeine, and brain injury severity. Subjects were asked to list any significant medical diagnoses, and any current medications, including psychotropics.

Behavioral and Cognitive Battery

All neuropsychological testing was administered in the UNM Center for Brain Recovery and Repair Clinical Core by trained study personnel under direct supervision of clinical neuropsychologists. The pre- and post-intervention assessments consisted of the following tests: the Neurobehavioral Symptom Inventory (NSI) (3); the Hamilton Depression Rating Scale (HAM-D) (46); the Beck Depression Inventory-II (BDI) (47); the Posttraumatic Stress Disorder Checklist-Civilian version (PCL-C) (48); the Patient-Reported Outcomes Measurement Information System-29 (PROMIS) (49); the Glasgow Outcome Scale-Extended (GOS-E) (50); the Frontal Systems Behavior Scale (FrSBe) (51); Wechsler Adult Intelligence Scale-Fourth Edition (WAIS-IV): Digit Span and Coding subtests (52); the Test of Premorbid Functioning (TOPF) (53); the Hopkins Verbal Learning Test-Revised (HVLT-R) (54); and Test of Memory Malingering (TOMM) (55). These tests were selected due to their inclusion in the NINDS Common Data Elements for TBI, as well as their history of validation in TBI populations. The NIH Executive Abilities: Measures and Instruments for Neurobehavioral Evaluation and Research (EXAMINER) battery was utilized as a more specific assessment of executive functions (56), with subscores of fluency, cognitive control, and working memory, as well as an overall executive composite score. Testing was performed at study entry (Baseline Visit), immediately after completion of the intervention (Post-Treatment visit), and 1 month after study entry (Followup Visit). To mitigate fatigue, testing was performed over 2 days, and regular breaks were offered, with total time of testing ∼5 h.

Intervention

Participants were randomly assigned to either active or sham tDCS combined with executive function training tasks. A NeuroConn tDCS device (neuroCare Group GmbH, Munich, Germany) was used to administer tDCS. Sessions consisted of 30 min stimulation for 10 consecutive weekdays. The anodal electrode was placed on the left dorsolateral prefrontal cortex (DLPFC; F3 position, International 10–20 system) utilizing the Beam F3 targeting method (57) and the cathode was placed on the right upper arm just below the deltoid muscle to isolate anodal cerebral effects (58, 59). Neuroconn 5 cm^2 rubber electrodes covered in 0.9% saline-soaked sponges were applied using elastic bandage. Current for the active condition was applied at 2.0 mA for a total delivered charge of 60 mA-min and a current density of 0.08 mA/cm^2, consistent with guidelines describing acceptable safety and blinding at this current density (60). Active stimulation current was ramped up over 1 min at initiation, maintained for 30 min, and ramped down over 1 min at termination. Sham stimulation was delivered with an initial ramping up of current to 2.0 mA for 1 min, then ramping down and remaining at 0.02 mA for the duration of the session, to permit impedance monitoring. Double-blinding of subjects and study staff was accomplished using pre-determined stimulation codes entered into the stimulator. During tDCS application, subjects were assessed in terms of tingling, itching, mood, energy, pain, and wakefulness levels using visual analog 10-point scales. Sensation checks were performed every 10 min during the stimulation session.

All participants were administered a set of executive functions training tasks for 30 min during stimulation sessions. Each training session consisted of 10 min of the AX Continuous Performance Task (AX-CPT), a test of response inhibition, proactive and reactive cognitive control (61), and 20 min of a modified multimodal (visual/auditory) N-back working memory task (MMWM) (62), counterbalanced over the 10 sessions. These tasks were selected based on their relevance to the three executive functions comprising cognitive control (working memory, response inhibition, set shifting) (63, 64) and prior studies of cognitive control in TBI (65, 66).

Cerebral Perfusion Imaging

MRI scans were performed during the baseline assessment visit, and on the day following completion of the stimulation protocol. MRI data was acquired on a 3T Siemens Trio scanner with a 32-channel head coil (see **Supplementary Methods**). High resolution T_1-weighted ($1 \times 1 \times 1$ mm), T_2-weighted ($1.1 \times 1.1 \times 1.5$ mm), susceptibility weighted images ($1.00 \times 1.00 \times 1.50$ mm) and fluid attenuated inversion recovery images ($0.80 \times 0.80 \times 3.00$ mm) were collected and reviewed by a blinded, board certified radiologist. Pseudo-Continuous Arterial Spin Labeling (pCASL; 45 tagged/untagged images) sequence was acquired (TR = 4,250 ms; TE = 11 ms; label offset = 90 mm; NEX = 1; slice thickness = 5 mm with 20% gap; bandwidth = 2,790

Hz/Px; labeling duration = 1,665 ms) with 20 interleaved slices for whole brain coverage (voxel size = 3.44 × 3.44 × 6.00 mm). A proton density (PD) sequence was also acquired to estimate T1 magnetization and scale CBF on a voxel-wise basis, with the post-labeling delay (PLD) and TR (5,200 ms) being the only parameters that varied across the pCASL (PLD = 1,800 ms) and PD scans (PLD = 3,400 ms).

To process pCASL for analysis, pCASL images were first despiked and registered to the pseudo-PD image using 2- and 3-dimension motion correction algorithms within the AFNI suite (67). Both images were then spatially blurred using a 6-mm Gaussian kernel. Each pre-processed labeled image was next subtracted from the paired control image, after which cerebral blood flow (CBF) was quantified using in-house software based on established parameters (blood/tissue water partition coefficient = 0.9 mL/g; longitudinal relaxation time of blood = 1,664 ms; labeling efficiency = 0.85; label duration = 1,665 ms) and algorithms (68). T1 magnetization correction and scaling of CBF was accomplished on a voxel-wise basis with the PD image. The quantified CBF data were then averaged and spatially transformed to standard stereotaxic space (69) using a non-linear transformation (AFNI 3dQwarp).

Data Analysis

All data were double entered and underwent quality assurance checks prior to statistical analysis. Sample size was determined based on previously reported Cohen's *d* effect sizes of 1.2 for tDCS to induce improvements in cognition using a similar unicephalic electrode montage (70). Sample size calculation given this effect size indicated 13 subjects per group would achieve 80% power to detect a difference at the 0.05 level. A series of mixed-models repeated measures ANOVAs were utilized to analyze the pre- and post-intervention data, with between-subjects factors of GROUP (2 levels) and SEVERITY (2 levels), and a within-subjects factor of VISIT (3 levels). Main effects F values were calculated for each within-group and between-group factor as well as an interaction effect. Primary outcome variables for imaging were: (1) Global CBF value; (2) Regional CBF values in regions in the AAL Atlas (71) with interest around the anode. False Discovery Rate (FDR) corrections for multiple comparisons were performed within each hypothesis for the primary outcome variables. Correlations between change in regional CBF, cognitive, and symptom variables were calculated and examined for trends. All statistical analyses were run on R v3.5.3 (R Core Team, 2019) (72).

RESULTS

Demographic Data

There were 10 subjects in the active tDCS group, and 14 subjects in the sham group, owing to the randomization protocol of the parent study. Baseline demographic variables, behavioral symptom scores, and neuropsychological test results are provided in **Table 1**. There were no significant baseline differences between the two groups.

TABLE 1 | Baseline average demographic, behavioral, and neuropsychological performance characteristics of the active and sham groups.

	Active (10)	Sham (14)	Sig (*p*)
Male/female	6/4	9/5	0.84
Mild/moderate	8/2	10/4	0.64
Tobacco	9	13	0.82
Caffeine	5	9	0.51
Age	29.4	36.8	0.15
Education	14.7	14.7	0.99
Hand laterality	75	94.6	0.29
GOSE	6.5	6.2	0.51
TOMM	46.1	46.7	0.77
TOPF	107	105	0.82
BDI	15.2	17	0.64
HAM-D	15.3	16.5	0.73
NSI-somatic	9.3	9.1	0.96
NSI-cognitive	7.4	5.9	0.41
NSI-emotional	9.7	8.9	0.73
PCL-C	40.2	39.3	0.88
PROMIS-physical	18.1	16.1	0.21
PROMIS-anxiety	10.2	9.9	0.84
PROMIS-depression	9.2	8.6	0.72
PROMIS-fatigue	12.1	10.6	0.33
PROMIS-sleep	13.8	12.6	0.45
PROMIS-social satisfaction	11.1	12.1	0.56
PROMIS-pain interference	8.7	10.1	0.53
PROMIS-pain intensity	3.1	3.4	0.82
WAIS-DS	11	9.5	0.33
WAIS-CD	10	10.3	0.84
HVLT-recall	44.7	41.8	0.52
HVLT-delayed	38.4	45.1	0.27
HVLT-retention	38.3	48.7	0.11
HVLT-discrimination index	40.2	48.6	0.11
FRSBE-apathy	68.1	68.9	0.92
FRSBE-disinhibition	62.8	60	0.63
FRSBE-executive dysfunction	71.7	66.7	0.49
FRSBE-total	71.3	68.6	0.73
Examiner working memory	0.77	0.68	0.67
Examiner fluency	0.54	0.9	0.28
Examiner cognitive control	0.5	0.68	0.57
Examiner executive composite	0.65	0.95	0.28

Neuropsychological Performance

There was a significant main effect of VISIT observed in multiple behavioral and neuropsychological variables after correction for false discovery rate (FDR), including the BDI, HAM-D, NSI somatic and emotional subscores, PCL-C, WAIS-CD, and Examiner composite and executive scores (F = 7.0–18.9, all $p < 0.01$) (see **Supplementary Table 1**). Depression, anxiety, and postconcussive symptoms all decreased over time from Baseline to Post-Treatment Visit, while complex attention and executive functions improved. There were no main effects of GROUP nor interaction effects of GROUP × VISIT for any variables (see **Figure 1**).

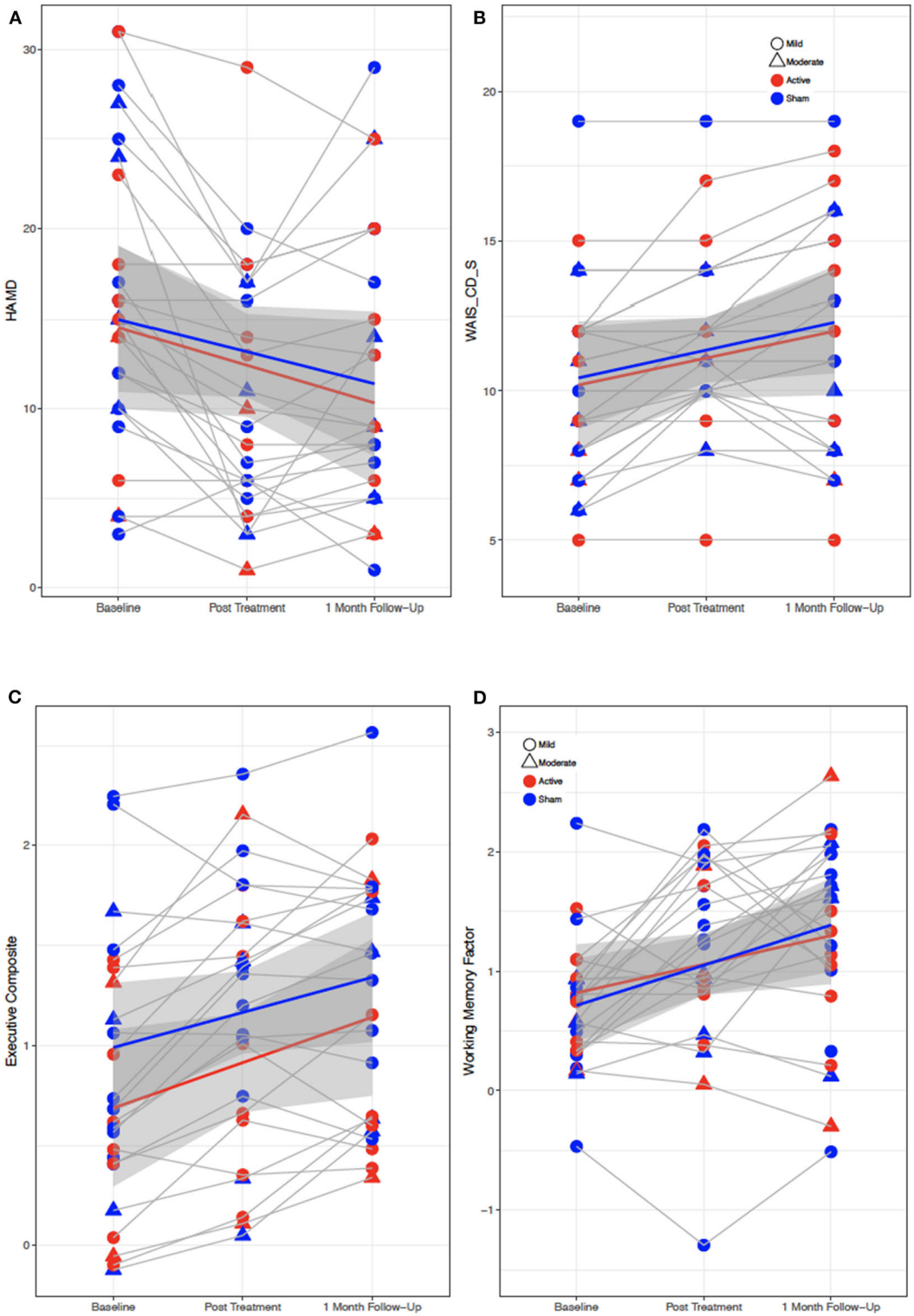

FIGURE 1 | Behavioral and cognitive performance for all subjects from baseline to post-treatment to 1 month followup visit. **(A)** Depression symptoms (HAM-D). **(B)** Attention performance (WAIS-CD-S). **(C,D)** Executive function performance (EXAMINER Executive composite and working memory composite scores). Red, active; blue, sham; gray regions, standard error.

Global Perfusion

Active and sham group mean values for global and regional CBF at Baseline and Post-Treatment time points are reported in **Supplementary Table 1**. A main effect of VISIT was observed, with a reduction of global CBF observed from Baseline to Post-Treatment Visit [$F_{(1, 23)} = 6.417$, $p = 0.02$] (see **Figure 2A**). While participants with moderate TBI had lower average perfusion values at both time points, the difference between subjects with mild vs. moderate TBI was not significant [$F_{(1, 21)} = 2.42$, $p = 0.14$] and VISIT*SEVERITY was not significant [$F_{(1, 22)} = 0.02$, $p = 0.89$]. Reduction in global CBF was weakly correlated with improvement on the HVLT Retention with $r = -0.44$, $p = 0.03$ (0.79) (FDR corrected p value in parentheses) (see **Figure 2B**).

Regional Perfusion

In the regional analysis of cerebral perfusion, active and sham groups demonstrated significantly different CBF changes in the inferior frontal gyrus (IFG) from Baseline to Post-Treatment Visits (see **Figure 3**). In the active group, perfusion remained static in the left IFG, and increased in the right IFG, while in the sham group, both left and right IFG demonstrated reductions in perfusion. Only in the right IFG was the difference between active and sham significant [$F_{(1, 22)} = 6.12$, $p = 0.02(0.984)$]. No regions passed FDR correction, and the only uncorrected regions with $p < 0.05$ were the cerebellum and the right IFG/pars triangularis.

To understand potential contributors to the observed CBF changes, an exploratory analysis was conducted of correlations between changes in right IFG CBF, neuropsychological test performance, TBI symptoms, and mood/anxiety/quality of life symptoms for both the active and sham groups (see **Supplementary Figures 1a,b**). In the sham group, mild to moderate correlations were observed between right IFG CBF and BDI/HAM-D ($r = 0.19$–0.45), NSI subscales ($r = -0.31$ to 0.52), PROMIS subscales ($r = -0.04$ to 0.48), WAIS-DS ($r = -0.37$), HVLT Retention scores ($r = -0.37$) and EXAMINER scores ($r = -0.08$ to -0.22). HAM-D was moderately correlated with performance on the HVLT Retention ($r = -0.42$), WAIS Digit Span ($r = -0.43$), and EXAMINER subscales ($r = -0.25$ to -0.55), while BDI tended to demonstrate correlations in the opposite direction.

In the active group, moderate to strong correlations were observed between right IFG CBF and NSI subscales ($r = 0.46$–0.85), BDI/HAM-D ($r = 0.56$–0.67), PROMIS subscales ($r = -0.31$ to 0.61), HVLT retention ($r = -0.69$), and FrSBe subscales ($r = 0.42$–0.75). Only mild and highly variable correlations were observed with the EXAMINER subscales ($r = -0.45$ to 0.3). A single outlier appeared to be driving most correlations, and excluding this data point reduced the strength of correlations to mild. Mild to strong correlations were found between depression scores (HAM-D, BDI) and NSI subscales ($r = 0.27$–0.78) and with performance on the HVLT Recall ($r = -0.43$ to -0.8) and Retention ($r = -0.17$ to -0.6).

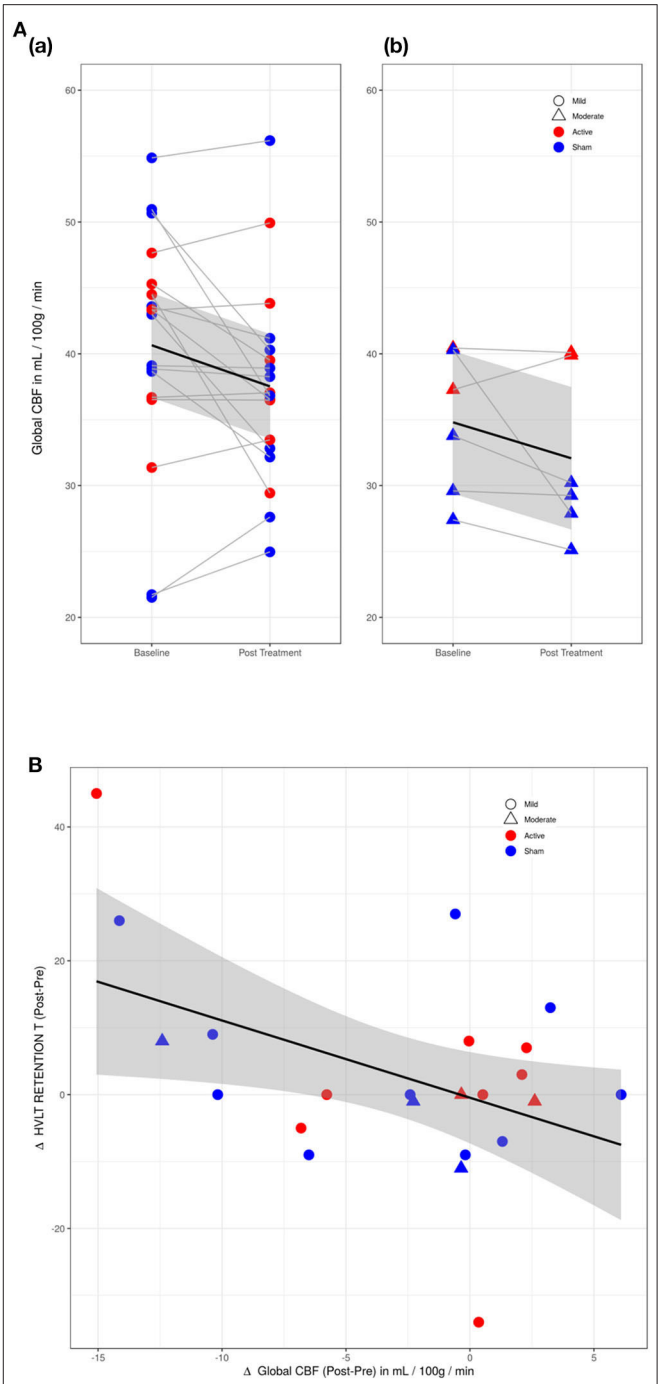

FIGURE 2 | (A) Changes in global cerebral perfusion (CP) between baseline and post-treatment visits. (a) Mild TBI participants (circles). (b) Moderate TBI participants (triangles). Difference in CP between mild and moderate TBI participants was not significant. Red, active group; blue, sham group. **(B)** Scatter plot of Hopkins Verbal Learning Test (HVLT) retention score with cerebral perfusion (CP) for active (red) and sham (blue) groups. HVLT correlated mildly ($r = -0.44$) with reductions in CP.

DISCUSSION

In this small randomized sham study of patients with chronic mmTBI and cognitive deficits, cerebral perfusion changes were noted following a cognitive training paradigm and anodal tDCS to the left dorsolateral prefrontal cortex. Global CBF was noted to decrease over time, while increases in mood, attention, and executive function were observed, consistent with a hypothesis

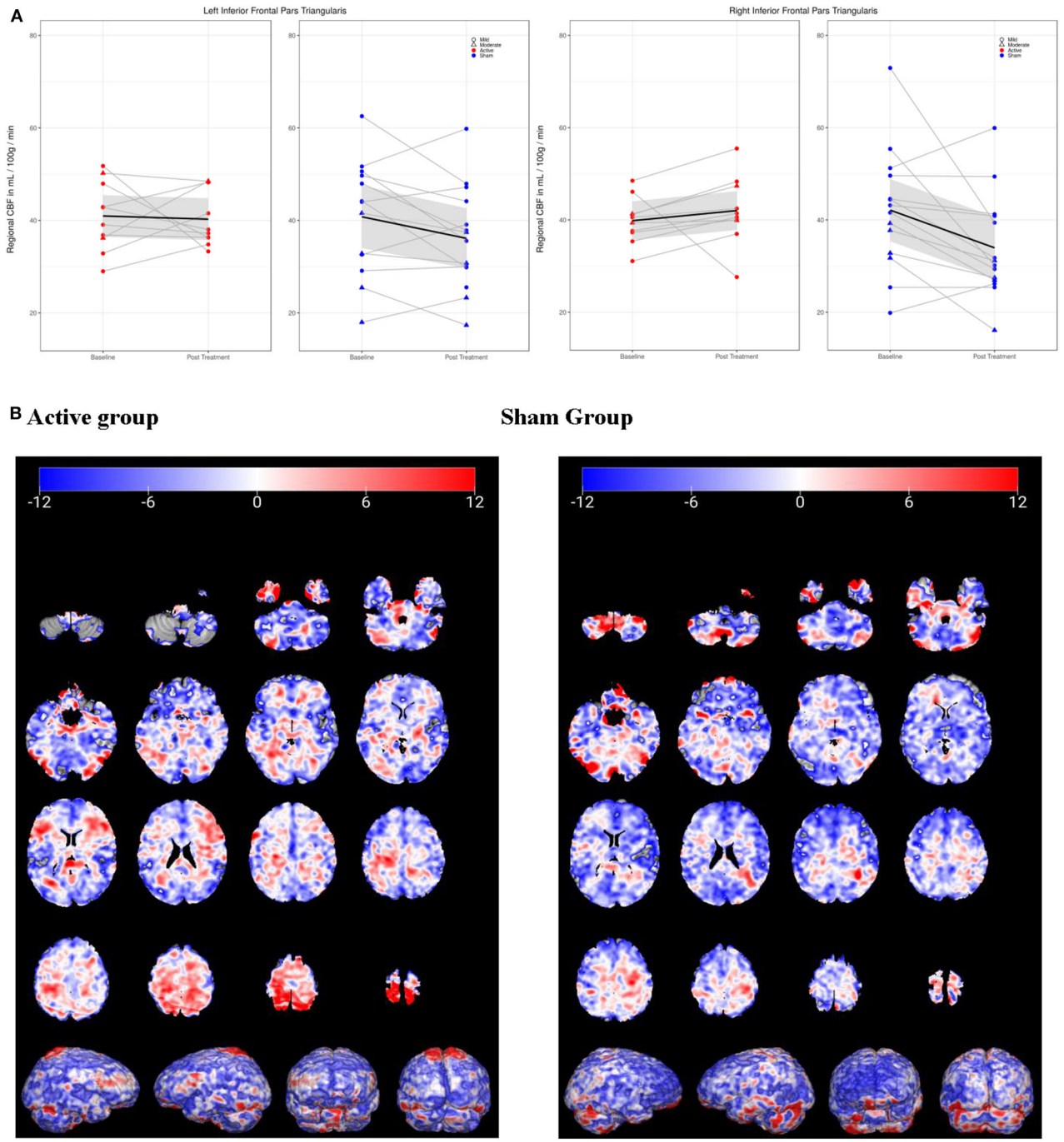

FIGURE 3 | Cerebral perfusion (CP) in inferior frontal gyrus/pars triangularis (IFG) from baseline to post-treatment visit. **(A)** In left IFG, active group (red) demonstrated no change in CP, while sham (blue) decreased. In right IFG, CP increased in active group but decreased in sham group. **(B)** 3-D visualization of change in CP over time in active and sham groups. Blue, decreased CP; red, increased.

of increased cerebral efficiency. Baseline CBF values in our study were similar to other studies reporting decreased global CBF following injury. Our population of mild-moderate TBI patients manifested, on average, a global initial CBF rate of ∼38–40 mL/100 g/min. This finding is consistent with that of previously cited studies, which found post-TBI regional CBF rates of 32–53 mL/100 g/min (26, 32, 33). However, changes in global CBF did not associate with objective cognitive performance or subjective mood measures, suggesting a more complicated relationship between clinical condition and generalized perfusion

than initially hypothesized. There was no additional effect of tDCS seen, with both groups demonstrating decreases in global CBF regardless of receiving active or sham stimulation. This is not necessarily surprising, given that tDCS is applied in a targeted manner to a specific cortical region, and prior studies indicate that tDCS-induced changes in perfusion occur in specific regions rather than across the entire brain (37, 40).

In the regional analysis of cerebral perfusion, an effect of tDCS was observed, with reductions of CBF occurring in the right IFG in the sham group, and increases of CBF in the right IFG of the active group. Although FDR correction resulted in nonsignificance, this finding is still of some interest, as there is theoretical and empirical basis for altered right prefrontal and right fronto-parietal perfusion after mTBI (32, 73–75). The right frontoparietal network is implicated in lateralization of cognitive and emotional functions, including inhibition (76), visual attention, and emotional sham. Right frontal dysfunction has also been associated with several symptoms such as depression (77), anxiety (78), somatization (79), impulsivity (80), and distractibility (81), all of which may be seen in chronic mmTBI.

The effect of anodal tDCS on regional CBF in this study was consistent with other studies of excitatory tDCS and cerebral blood flow, in that an increase in perfusion was observed in the prefrontal cortices where current density is predicted to be highest (82). The larger CBF effect being observed in the right IFG as opposed to under the electrode on the left is somewhat paradoxical but may be explained by the strong functional connectivity typically observed between cortical regions and their homolog in the opposite hemisphere (e.g., interhemispheric transfer) (83, 84). While this regional finding is encouraging for tDCS having a potentially beneficial effect on perfusion and chronic symptoms of mmTBI, it did not correlate significantly with neuropsychological performance nor with subjective symptom report following treatment. There is a theoretical concern that if beneficial effects of cognitive training relate to reduced regional CBF, anodal tDCS may actually be counterproductive or deleterious to this process. However, the exploratory correlation analysis did not support this possibility, as correlation strengths between CBF and symptom/performance improvements in the sham and active group were approximately the same. Also of note in the exploratory analysis was that of the strongest correlations found were between emotion measures and executive function performance in both groups, pointing to a potential nonspecific mood benefit of the training or study protocol that may have obscured any contribution from the tDCS.

We consider our finding of decreased global and regional perfusion following the cognitive intervention somewhat paradoxical, considering that our study sample was already manifesting reduced CBF values at baseline. This raises the theoretical question of how decreased CBF can be both a marker of injury, as well as a marker of rehabilitation response. While it is possible that global CBF may have decreased over time independent of injury status or the intervention, CBF measured by pCASL has been shown to be relatively stable over time (85). Another possibility is that lower CBF is an adaptation to the injured state, and that with training, the adaptation is strengthened or amplified. Barlow et al. found that recovered patients after concussion manifested lower CBF values than controls, suggesting that recovery does not necessarily involve a return to original baseline CBF values (30). A second possibility is that while global CBF may be an accurate reflection of an injury condition, the recovery process may be occurring at a more regional level. This is suggested by our asymmetric CBF findings following tDCS and training: right frontal decreased perfusion, accompanied by left frontal increased perfusion, may be a recovery pattern for mood, cognition, and behavior, such as is seen in transcranial magnetic stimulation for depression (86, 87). A final consideration is that resting CBF measured by pCASL may fail to fully account for changes to dynamic cerebrovascular regulation and its relation to metabolic demand. Static reductions in cerebral perfusion after TBI may be accompanied by increases in CBF with effort on functional sequences such as task-based fMRI or cerebrovascular reactivity challenges (88, 89). It might be necessary to obtain measurements of both CBF and metabolic activity (i.e., positron emission tomography) in order to better understand how the brain is adapting to injury and responding to training (90). In summary, our findings suggest that the role of cerebral perfusion in the pathogenesis of and recovery from PPS continues to be complex.

This study was limited by its small sample size, uneven group numbers, heterogeneity of the clinical sample, and the lack of a non-treatment control group, limiting any conclusions that can be drawn. However, many patients with TBI fail to demonstrate any cognitive gains in rehabilitation, whereas this sample demonstrated robust improvements in multiple cognitive domains. Therefore, the prospect of a simple 10-day program of cognitive training on executive functions leading to objective improvements is cause for further study. Cerebral perfusion measured with pCASL represents a potential pathophysiologic target for rehabilitation paradigms in mmTBI such as cognitive training and tDCS.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by UNM HSC HRRC. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DQ was responsible for primary writing, literature review, and conceptualization of the study. JU, TJ, and AM were responsible for higher-level analysis of imaging and behavioral data. EB, JS-R, VF, RR, JW, and DG were responsible for data collection, curation, and primary analysis. MH, VC, RC, RY, CS, and JR provided input on study design, data collection methods, cognitive training, and stimulation protocols. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.545174/full#supplementary-material

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Neural Connectivity Changes Facilitated by Familiar Auditory Sensory Training in Disordered Consciousness: A TBI Pilot Study

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For people with disordered consciousness (DoC) after traumatic brain injury (TBI), relationships between treatment-induced changes in neural connectivity and neurobehavioral recovery have not been explored. To begin building a body of evidence regarding the unique contributions of treatments to changes in neural network connectivity relative to neurobehavioral recovery, we conducted a pilot study to identify relationships meriting additional examination in future research. To address this objective, we examined previously unpublished neural connectivity data derived from a randomized clinical trial (RCT). We leveraged these data because treatment efficacy, in the RCT, was based on a comparison of a placebo control with a specific intervention, the familiar auditory sensory training (FAST) intervention, consisting of autobiographical auditory-linguistic stimuli. We selected a subgroup of RCT participants with high-quality imaging data (FAST $n = 4$ and placebo $n = 4$) to examine treatment-related changes in brain network connectivity and how and if these changes relate to neurobehavioral recovery. To discover promising relationships among the FAST intervention, changes in neural connectivity, and neurobehavioral recovery, we examined 26 brain regions and 19 white matter tracts associated with default mode, salience, attention, and language networks, as well as three neurobehavioral measures. Of the relationships discovered, the systematic filtering process yielded evidence supporting further investigation of the relationship among the FAST intervention, connectivity of the left inferior longitudinal fasciculus, and auditory-language skills. Evidence also suggests that future mechanistic research should focus on examining the possibility that the FAST supports connectivity

changes by facilitating redistribution of brain resources. For a patient population with limited treatment options, the reported findings suggest that a simple, yet targeted, passive sensory stimulation treatment may have altered functional and structural connectivity. If replicated in future research, then these findings provide the foundation for characterizing the unique contributions of the FAST intervention and could inform development of new treatment strategies. For persons with severely damaged brain networks, this report represents a first step toward advancing understanding of the unique contributions of treatments to changing brain network connectivity and how these changes relate to neurobehavioral recovery for persons with DoC after TBI.

Clinical Trial Registry: NCT00557076, The Efficacy of Familiar Voice Stimulation During Coma Recovery (http://www.clinicaltrials.gov).

Keywords: attention, consciousness disorders, language, traumatic brain injury, white matter

INTRODUCTION

Coma recovery after traumatic brain injury (TBI) is described by degrees of consciousness delineated clinically as the vegetative state (VS), minimally conscious state (MCS), and emergence from MCS (1–4). These classifications represent a gradient of clinical consciousness where less consciousness is associated with more disruption of functional and structural neural connectivity (5–18). Recovery, however, is not necessarily a linear progression along this gradient (10, 19–21). Cross-sectional evidence suggests that behavioral recovery is supported by dynamic changes in hypoconnectivity and hyperconnectivity of neural networks local to and remote from lesion topography (8, 22–28). Emerging longitudinal evidence (29) also suggests that behavioral recovery (30) is supported by non-linear changes in hyporesting and hyperresting state functional neural connectivity (10, 19, 20, 31, 32). Collectively, the evidence suggests that changes in neural connectivity could precede or occur in parallel with neurobehavioral recovery.

Advancing knowledge of how changes in neural connectivity relate to neurobehavioral recovery is important, in part, because it will allow for identification of the unique contributions of specific interventions to brain and behavior relationships. To start building a body of evidence regarding the contributions of specific interventions to changes in neural network connectivity and the relationship of these changes to neurobehavioral recovery, we conducted a *post hoc* pilot study using a systematic approach to examine previously unpublished neural connectivity data from a double-blind randomized clinical trial (RCT) (33). As this RCT demonstrated the therapeutic efficacy of the familiar auditory sensory training (FAST) intervention, this longitudinal dataset enabled an examination of the unique contributions of a specific intervention, the FAST. Considering that usual care was paired, by random assignment, with either the FAST intervention or the placebo intervention, the RCT dataset also provides the basis to identify changes in neural connectivity specific to the FAST vs. changes related to differences in usual care practices (34, 35) and/or placebo effects (36). The RCT design also allows for the accounting of injury heterogeneity (etiologies, neuropathology, and secondary brain damage) in measures of change in neural connectivity.

The purpose of this article is to report pilot study findings of changes in neural connectivity related to the FAST intervention for persons remaining in states of disordered consciousness (DoC) after TBI. To the best of our knowledge, for this population, this article represents the first report of longitudinally based profiles of neural network connectivity changes relative to neurobehavioral recovery in response to a specific therapeutic intervention. For this scientifically challenging patient population, the reported study also demonstrates a systematic approach to explicating the relationships among neurobehavioral recovery and changes in neural connectivity of the broad neural networks thought to be targeted by the autobiographical auditory-linguistic stimuli used in the FAST intervention (37) (also see **Supplement A**): the language network (LN), salience

Abbreviations: AF, arcuate fasciculus; AN, attention network; BL, baseline; BOLD, blood oxygen level–dependent; CNC Scale, Coma–Near-Coma Scale; DARTEL, Diffeomorphic Anatomical Registration Through Exponentiated Lie algebra; a suite of tools for more accurate intersubject registration of brain images; DMN, default mode network; DoC, disordered consciousness; DOCS-25, Disorders of Consciousness Scale-25 (2014 version with 25 calibrated items); DTI, diffusion tensor imaging; DVARS, D = temporal derivative of the time course; VARS = root mean square of the variance over voxels framewise displacement relative to the root mean square signal change; spatial standard deviation of successive difference image; eMCS, emergence from minimally conscious state; EP, endpoint; EPI, echo planar images; F, FAST (F) group; FA, fractional anisotropy; FAST, familiar auditory sensory training; FD, framewise displacement; FDR, false discovery rate; FOV, field of view; FSL, FMRI Software Library; IFOF, left inferior fronto-occipital fasciculus; ILF, inferior longitudinal fasciculus; LN, language network; MCS, minimally conscious state; MFRM, multi-faceted Rasch measurement; MLM, mixed linear effects models; MNI152, Montreal Neurological Institute 152 template; Ordered p, independent sample permuted *t*-tests conducted with mean estimated *z* values; RCT, randomized clinical trial; ROI, region of interest; rsFC, resting state functional connectivity; rVLPFC, right ventral lateral prefrontal cortex; SD, standard deviation; SFOF, superior frontal-occipital fasciculus; SLF, superior longitudinal fasciculus; SN, salience network; TBI, traumatic brain injury; TE, echo time; TR, repetition time; UF, uncinate fasciculus; VS, vegetative state; *Z*, mixed-effects linear model estimates.

network (SN), attention network (AN), and default mode network (DMN) (**Figure 1**). The reported findings and scientific approach used to discover and identify relationships warranting further study, together, provide guidance for future research examining the unique contributions of the FAST intervention, as well as other interventions to neurobehavioral recovery from DoC after TBI.

MATERIALS AND METHODS

The present study is based on data for a subgroup of FAST RCT participants. The previously published RCT methods (33) relevant to the present study as well as procedures unique to the present study are provided here. The RCT was approved by the human subjects' institutional review boards at each study site. Informed consent was obtained from each participant's legally authorized representative.

The imaging subgroup was selected from the total FAST RCT sample. RCT participants were recruited from a Veterans Administration Inpatient Polytrauma Rehabilitation program, the Shirley Ryan Ability Lab inpatient rehabilitation program, and from community residences in a large urban area [see Pape et al. (33) for additional details]. To be eligible for the RCT, participants were required to (a) be 18 years or older, (b) have incurred a severe TBI within the previous year, and (c) be in a state of DoC for at least 28 days as a result of the TBI. Persons dependent on a ventilator and remaining in states of DoC due to non-traumatic or penetration injuries were not eligible. Randomization was stratified by each of the three study settings. Sixteen participants were randomized to the FAST or placebo groups, 15 of whom completed the RCT [mean age at injury = 35.1, standard deviation (SD) = 11.0; mean days after TBI = 69.8, SD = 42.8; male = 80%; MCS = 67%].

Neurobehavioral Outcomes

The primary and secondary neurobehavioral outcomes for the FAST RCT were the Disorders of Consciousness Scale-25 (DOCS-25) (38) and the Coma–Near-Coma (CNC) scale (39), respectively. As summarized in **Supplement C**, the DOCS-25 yields a reliable and valid measure (40) of best global overall multimodal neurobehavioral functioning, and it includes an Auditory-Language subscale. As this subscale measure was not the primary or secondary RCT outcome, the DOCS-25 Auditory-Language subscale measures were not previously reported (33). These subscale measures were included in the present study because they are specific and relevant to neural networks involved in language function and processing thought to be targeted with the FAST stimuli (37) (also see **Supplement A**). The CNC total score is a measure of arousal, attention, and awareness (41–43); lower scores reflect more consistent behavioral responses (39, 44).

Baseline (BL) and endpoint (EP) neurobehavioral tests were obtained 24 h after complete cessation of pharmaceutical CNS stimulants for all participants. The DOCS-25 was repeated weekly, and the CNC was repeated twice weekly (also see **Figure SC1** in Supplement C).

Image Acquisition

Anatomical, resting state, and diffusion tensor imaging (DTI) data were acquired at BL (prior to starting assigned intervention) and again at treatment EP. For this multisite RCT, all scans were acquired on two different 1.5-T scanners with a standard 12-channel head coil. Foam cushions and earplugs were used to reduce motion and scanner noise. Scanners were cocalibrated using previously described procedures [see **Supplementary Material** in Pape et al. (33)]. For the present study, however, all participants were scanned on the same magnet (i.e., Siemens Avanto).

For functional magnetic resonance imaging (MRI) resting state acquisition, 205 whole-brain T2*-weighted echo planar images (EPIs) were acquired in a 10-min, 15-s scan, with a repetition time (TR) of 3 s; echo time (TE) of 40 ms; and a flip angle of 90°, with a 64 × 64 matrix, a field of view (FOV) = 220 × 220 mm, and a voxel size of 3.4 × 3.4 × 3.0 mm^3. For registration purposes, a high-resolution anatomical T1-weighted sequence was acquired at BL and, considering potential morphological changes following treatment, was also collected at EP. Both were collected using three-dimensional (3D) magnetization-prepared rapid gradient-echo sequence with the following parameters: 176 slices, TR/TE/flip angle = 2,400 ms/3.72 ms/8°, inversion time (TI) = 1,000 ms, voxel size = 1 × 1 × 1 mm^3, FOV = 256 mm.

DTI data were acquired using a spin-echo EPI sequence with the following parameters: TR/TE/flip angle = 6,000 ms/89 ms/90°; 30 gradient orientations with b = 1,000 s/mm^2, three images with b = 0, FOV = 220 × 220 mm with a matrix size of 144 × 120 leading to an in-plane resolution of 1.77 × 1.77 × 3 mm^3.

Functional Imaging Preprocessing

Resting state EPI data were preprocessed using SPM8 in MATLAB R2012b. The first three volumes of the 205 acquired were discarded for MRI signal stabilization. The remaining 202 volumes were realigned to the first EPI volume. To preserve anatomical injuries and reduce spatial variability between subjects to enable group comparisons, the voxel-based morphometry toolbox was used for the non-linear warping to normalize the best T1 image using DARTEL (Diffeomorphic Anatomical Registration Through Exponentiated Lie algebra; see **Figure SC2** in Supplement C for non-linear warping explanation and illustration) from BL and EP to the Montreal Neurological Institute 152 template (MNI152). BL and EP resting state data were linearly warped to the native T1 using SPM. Because of lesion type and location variability between subjects, a region of interest (ROI)–based manual segmentation was performed for each participant. Blood oxygen level–dependent (BOLD) data were detrended and bandpass filtered (0.01–0.08 Hz). The white matter mask for resting state data covers normal-appearing white matter superior to the lateral ventricle. The cerebral spinal fluid mask for resting state data covers the middle section of the lateral ventricle farthest from the gray matter boundary. While global signal regression has been shown to reduce motion effects in resting state data, applying global signal regression is controversial as it has also been shown to remove some valuable neural signal and to alter the BL reference (45). Therefore, we

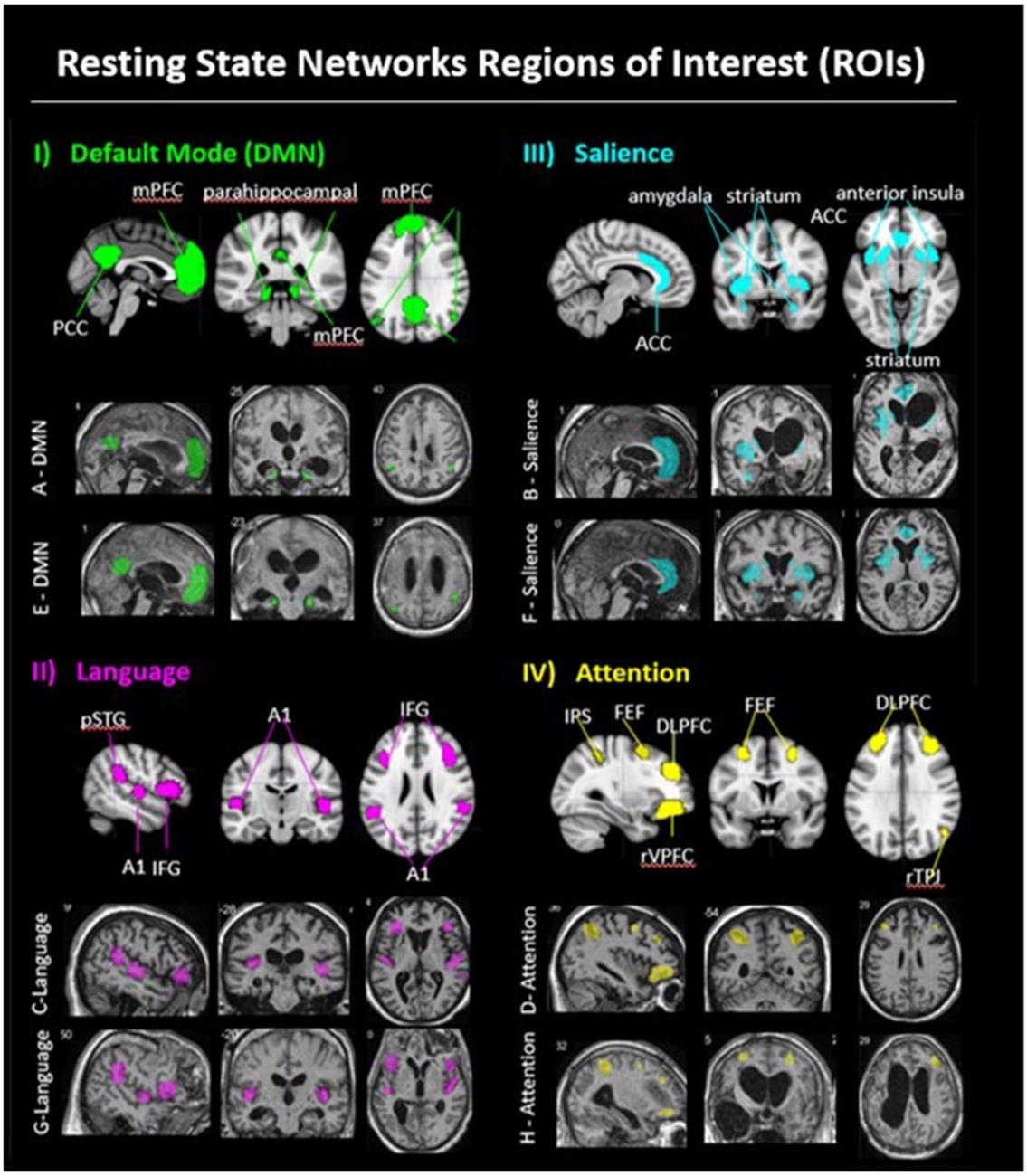


FIGURE 1 | Resting state networks regions of interest (ROI). (i) DMN: Panel I, row 1 shows a DMN exemplar on the MNI 152 standard brain, with medial prefrontal cortex (mPFC), posterior cingulate cortex (PCC), bilateral parahippocampal area, bilateral temporal parietal junction (TPJ). Panel I, row two shows example of a FAST subject's DMN ROIs (patient A); panel I. row 3 shows an example of a placebo subject's DMN ROIs (patient E). (ii) Language: Panel II, row 1 shows the language network ROIs on the MNI 152 standard brain, with bilateral posterior superior temporal gyrus (pSTG; Wernicke area), bilateral primary auditory cortex (Heschl gyrus), and bilateral inferior frontal gyrus (IFG; Broca area). Panel II, row 2 shows an example of a FAST subject's language ROIs (patient C); panel II, row 3 shows an example of a placebo subject's language ROIs (patient G). (iii) Salience: Panel III, row 1 shows the salience network ROIs on the MNI 152 standard brain, with anterior cingulate cortex (ACC), bilateral amygdala, bilateral anterior insula, and bilateral striatum. Panel III, row 2 shows an example of a FAST subject's salience ROIs (patient B); panel III, row 3 shows an example of a placebo subject's salience ROIs (patient F). (iv) Attention: Panel IV, row 1 shows the attention network ROIs on the MNI 152 standard brain, including bilateral dorsal lateral prefrontal cortex (DLPFC), bilateral frontal eye fields (FEF), bilateral intraparietal sulcus (IPS), right temporal parietal junction (rTPJ), and right ventral lateral prefrontal cortex (rVLPFC). Panel IV, row 2 shows an example of a FAST subject's attention ROIs (patient D); panel IV, row 2, shows an example of a Placebo subject's attention ROIs (patient H).

ran a binary regression on each volume with motion regressor thresholds set to the temporal derivative of the time course (DVARS, where VARS = RMS of the variance over voxels), for DVARS > 50 (i.e., 5% of BOLD signal) and framewise displacement (FD < 0.5 mm) as thresholds (46). Following the binary regression, one participant had only 108 remaining resting state volumes. To enhance comparability between subjects, for each subject the first 108 volumes that survived the binary regression thresholds were used for analyses (≈5 min and 24 s of resting state data per subject).

DTI Preprocessing

DTI data were processed using the FMRI Software Library (FSL) Diffusion Toolkit (FDT; http://www.fmrib.ox.ac.uk/fsl). The brain extraction tool was applied to the first non-diffusion-weighted B0 image for skull stripping, and a mask was created from the skull-stripped volume. FDT diffusion was used for eddy current and motion correction. DTIFIT was used to create voxelwise diffusion tensor models within the whole-brain mask to produce fractional anisotropy (FA) maps. The longitudinal data were not normalized to a standard template. FLIRT utilities were used to create halfway transforms to apply the same level of transformation (interpolation) to BL and EP data for each subject. The EP b0 image was registered to the BL b0 image using an Affine 12 parameter model. The command "avscale" was run on the EP-to-BL registration output matrix to generate a "halfway forward" and "halfway backward" transformation matrix. The halfway backward transform was then applied to EP FA maps, and the halfway forward transform was applied to the BL FA maps. FSLmaths was used to binarize and multiply the FA maps from both timepoints, to produce a mask of common voxels, with a threshold of 0.2 to include partial-volume edges.

Imaging Subgroup Selection

The FAST RCT included a total of 15 participants, with 14 being scanned on the same magnet (i.e., Siemens Avanto). Of these 14 participants, six were excluded from the imaging subgroup. The primary reason for exclusion was motion that, after motion reduction procedures conducted during preprocessing, exceeded 3 mm (also see consort diagram, **Supplement B**). For the remaining eight participants, motion was also examined according to within-subject differences between BL and EP. To identify motion differences, we conducted a series of two-tailed non-parametric Wilcoxon signed rank tests for paired BL and EP measures of the FD and the temporal derivative of the time course (DVARS, where VARS = RMS of the variance over voxels) and found no significant differences [FAST FD BL ($n = 4$) mean = 0.42, SD = 0.36 vs. EP ($n = 4$) mean = 0.66, SD = 0.30, $p = 0.10$; FAST DVARS BL ($n = 4$) mean = 37.73, SD = 6.35 vs. EP ($n = 4$) mean = 36.27, SD = 6.41, $p = 0.86$; placebo FD BL ($n = 4$) mean = 0.66, SD = 0.40 vs. EP ($n = 4$) mean = 0.78, SD = 0.65, $p = 0.86$; placebo DVARS BL ($n = 4$) mean = 53.03, SD = 15.01 vs. EP ($n = 4$) mean = 41.93, SD = 10.23, $p = 0.36$]. Thus, the imaging subgroup for the present study includes eight RCT participants scanned on the same magnet with imaging data of sufficient quality ($n = 4$ from FAST group and $n = 4$ from the placebo group; **Table 1**).

Regions of Interest

For the eight participants in the present study, resting state functional connectivity (rsFC) analyses were conducted using a total of 26 ROIs, which were manually drawn by a single researcher on the normalized T1 (**Figure 1**) in MRICron (47). Regions were referenced against gray matter atlases (48, 49). For six of the eight participants, ROIs were drawn on the BL normalized T1 and applied to the coregistered data at EP. The two remaining participants, however, had substantial morphological changes at EP (i.e., reduced subdural hematoma; cranioplasty); thus, ROIs were separately hand-drawn for those two participants on the normalized EP T1 and BL normalized T1.

Given anatomical variability between subjects, an ROI-based manual segmentation method was also implemented for DTI analyses. All 19 white matter tracts were hand-drawn for each participant by a single researcher. To minimize error and to exploit fiber orientation, tracts were drawn on each coronal slice of the BL 2D FA maps using the b0 images as the primary anatomical reference. Each tract was drawn in its entirety, from the largest to the smallest diameter sections, while being referenced against the ICBM-DTI-81 white-matter labels atlas in FSL. ROI verification and refinements were made in the sagittal and axial planes (50, 51). The whole-brain mask of common voxels was then multiplied by each white matter ROI to isolate ROI specific voxels, present at both timepoints.

Computation of Brain Network Connectivity Metrics

To compute rsFC metrics, a time series of the resting state BOLD signal was extracted for each of the 26 ROIs using the MATLAB REST toolbox. If an ROI was not discernible or not present, then the time series was not imputed and was classified as missing data. For rsFC network analyses, we then used two distinct approaches to compute brain connectivity metrics indicative of communication within-networks and between-networks. For within-network analyses, we computed rsFC metrics using an approach that preserves ROI–ROI correlation strengths. For between-network connectivity analyses, we computed rsFC metrics using a network masking approach that preserves signal strength.

To address the potential for broad dysregulation within an individual network, we used an ROI–ROI correlation approach to compute the rsFC metrics for use in within-network analyses. This approach preserves correlation strengths across each ROI including the anticorrelations and differences in ROI–ROI correlation strengths. Specifically, this approach uses ROI–ROI correlation strengths to characterize the synchrony within an individual network.

After generating a 26 × 26 pairwise ROI–ROI correlation matrix, (52) the Fisher z transformation was applied. We calculated rsFC of each individual network (DMN, LN, SN, AN) by averaging the ROI–ROI Pearson correlations z scores within a network and generated within-participant and group-level (FAST, placebo) means.

To assess between-network connectivity, we used an approach to computing rsFC metrics that preserves signal strength (rather than correlation strength) to weigh the contribution of each node. This analytic strategy yields a better reflection of between-network communication where any strong node can drive the network–network communication. For each network, a mask was created by averaging the time series for each ROI (i.e., rather than averaging individual ROI–ROI correlations). A distinct advantage of this approach is that it preserves signal strength (over correlation strength), thereby preserving the contribution of dominant nodes. Thus, the use of signal strength as an index of between-network communication allows for any strong node to drive the network–network communication. For example, a very strong signal may be anticorrelated with a very weak signal; the correlation is strong, but the contribution from each node is unequal. Conversely, two weak anticorrelated signals may produce similar correlation strength. Averaging the signals preserves the contribution of the dominant signal while averaging out the weak anticorrelations.

To calculate between-network rsFC, the time series for each ROI in a network was extracted and then averaged at the network level (e.g., for LN: L Broca + R Broca + L Heschl + R Heschl + L Wernicke + R Wernicke/6). The resulting network time series for the DMN, LN, SN, and AN were then used to generate a Fisher z-transformed 4 × 4 correlation matrix for each participant. Group-level (FAST, placebo) means were calculated.

For DTI metrics, we examined FA that is a reliable metric that self-normalizes between 0 and 1 (53). We used fslstats in the FSL toolkit to extract mean FA values and SDs for each ROI. Group-level (FAST, placebo) mean FA values were then calculated for each timepoint (54).

Computing Neurobehavioral and Neural Connectivity Measures for Use in Data Analyses

The raw neurobehavioral scores and brain connectivity metrics were used to compute equal-interval measures according to a process of precision optimization. For neurobehavioral measures, this computational process started with transforming raw scores using Rasch measurement models because this enhances precision of each patient's estimated neurobehavioral function (55, 56). The DOCS-25 Total and DOCS-25 Auditory-Language subscale scores were transformed using multifaceted Rasch measurement (MFRM) (57–59). The MFRM approach enhances measurement precision by neutralizing between-rater differences and by attenuating within- and between-subject variability not related to neurobehavioral function (40, 57–59). Because CNC test items each have a unique rating scale, CNC raw scores were transformed using a partial-credit Rasch model (60) as it accounts for differing scales.

The Rasch transformed DOCS-25 Total, DOCS-25 Auditory-Language, and CNC measures and all brain network connectivity metrics were used in mixed-effects linear models (MLMs). The MLM approach was used because it produces robust parameter estimates by borrowing strength from each and every measure, accounting for interdependency of repeated observations and by accounting for within- and between-subject variability (61). Specifically, MLM was used to estimate model parameters, the intercept (β_1), slope (β_2), and both random effects (see **Supplement C** for additional details on MLM modeling procedures).

TABLE 1 | Pilot study imaging Subgroup demographics.

	Total ($n = 8$)	FAST ($n = 4$)	Placebo ($n = 4$)
Mean age at injury (years)	39.6, SD = 12.3	40.5, SD = 13.7	38.8, SD = 12.7
Mean days after TBI	77.9, SD = 37.6	77.8, SD = 38.2	78.0, SD = 42.9
Percent male	0.88	0.75	100
Percent VS baseline	0.38	0.50	0.25
Percent MCS baseline	0.62	0.50	0.75
Percent VS endpoint	0.25	0.25	0.25
Percent MCS endpoint	0.50	0.25	0.75
Percent eMCS endpoint	0.25	0.50	0.00

MCS, minimally conscious state; eMCS, emerged from MCS to full consciousness; SD, standard deviation; VS, vegetative state.

For each MLM of the DOCS-25 Total, DOCS-25 Auditory-Language, and CNC measures, the precision of MLM model parameter estimates was enhanced by including the Rasch transformed neurobehavioral measures for all 15 RCT participants (62). For the DOCS-25 Total and DOCS-25 Auditory-Language models, each MLM included six timepoints per participant. For the CNC model, 12 timepoints per participant were included. After MLM, the neurobehavioral measures for the eight participants in the imaging subgroup were extracted from MLM results and retained for data analyses.

For MLM of the brain network connectivity metrics, each of the eight subgroup participants was used in the models. Fisher z-transformed metrics for each gray matter ROI and BL mean FA metrics were used in MLM (62). Two timepoints (BL and EP) were included in each model.

All MLM-derived neurobehavioral and brain connectivity measures, prior to conducting analyses, were verified by computing mean absolute error (MAE) and mean absolute relative error (MARE). The MAE and MARE identify the average error of the MLM estimated measures by BL, EP, and change from BL to EP.

Accounting for Missing Substrates in Brain Network Connectivity Metrics

Because of the uniqueness of the neuropathology among participants, each individual's own normalized image was used to represent their brain injury. Using this approach, the Fisher z-transformed values followed an approximately normal distribution. However, each participant had at least one gray matter region and/or white matter tract that was not clearly discernible or not present. Therefore, MLM was used to predict missing imaging values for indiscernible or non-present regions for a given participant. To predict these missing values, MLM uses the actual values and random effects of the other subjects in the model. This means that the predicted estimate is an average of other participants nested in the same group (FAST or placebo). The MLM estimated parameters were then imputed for the missing brain network connectivity metrics.

The MLM imputation procedures were validated by examining discrepancies between the observed metric and the estimated parameters using χ^2 tests (see **Supplement C** for additional details on validation procedures). After these validation procedures, the MLM parameter estimates of missing brain connectivity metrics were used for all brain connectivity analyses.

Data Used in Analyses: Indices of BL, EP, and Change

The MLM-derived neurobehavioral and brain connectivity measures, after verifications, were used in all data analyses. Each participant's predicted MLM parameters from each neurobehavioral and brain connectivity model were used to compute their BL (intercept) and EP [intercept + random intercept + (random slope + fixed slope) × time] neurobehavioral and brain connectivity measures. These computations of DOCS-25 Total, DOCS-25 Auditory-Language, CNC, rsFC, and FA were then used for all analyses as each patient's BL, EP, and change from BL to EP in neurobehavioral function and neural network connectivity.

To determine the influence of using imputed MLM parameters estimates for missing imaging metrics on revealing potentially important relationships, all analyses were repeated using the raw imaging metrics (i.e., see **Tables SD1–3** in Supplement D for all raw/unmodeled *z*, FA values).

Data Analyses: Examinations of Imaging Subgroup Representativeness

To identify potential sources of bias from the non-randomized selection of participants for this pilot study, analyses were conducted to examine the representativeness of the imaging subgroup to the RCT sample. Representativeness was examined by comparing (a) FAST RCT participants included in the imaging subgroup ($n = 4$) vs. FAST RCT participants excluded from the imaging subgroup ($n = 4$); and (b) placebo RCT participants included in the imaging subgroup ($n = 4$) vs. the excluded placebo RCT participants ($n = 3$).

The examinations of representativeness included comparisons of the MLM-predicted estimates of DOCS-25 Total, DOCS-25 Auditory-Language, and CNC measures of neurobehavioral function, as well as rsFC and DTI measures of brain connectivity. These examinations also compared injury severity, prognostic factors, demographics, and usual-care services. Differences in neurobehavioral measures were identified using 2-tailed Student *t*-tests. We also examined MLM derived trend lines.

Data Analyses: Discovering and Identifying Robust Relationships

The process of discovering relationships and then identifying those that warrant further study involved significance testing. Significance testing was not used to establish the extent of the intervention effects. Considering the magnitude of the data and the goal of identifying relationships warranting further investigation, significance testing was used to filter the relationships revealed in discovery procedures, thereby identifying robust relationships. Significance testing was conducted (unless otherwise specified) using non-parametric permutation statistical testing as it is flexible and robust under model violations. This approach is also powerful for small samples in which verification of the distribution of the test statistic is unreliable (63). For permutation tests, no specific distribution of the test statistic under the null hypothesis is assumed. Test statistics were obtained by calculating all possible values of test statistics under rearrangements of the labels on the observed data points. The exact ordered *p*-values for the permutation tests were computed using the number of ordered statistics greater than the original test statistic, t1, and the total number of permuted test statistics. For example, when $n = 4$ subjects, and there are 3 ordered test statistics > t1, the test will have an ordered $p = 3/2^4$ (i.e., = 3/16 where ordered $p = 0.19$). As multiple tests were involved with the significance testing (e.g., 10 tests for 10 within- and between-networks), we adjusted all ordered *p*-values by controlling the false discovery rate (FDR) using the Benjamini and Hochberg procedure with $q = 0.20$ (64) (see **Figure SC3** in Supplement C for additional details and an illustration). We opted for a liberal FDR to avoid the possibility of missing important relationships that warrant further examination in future research.

Non-parametric significance testing procedures were also used to identify BL differences between FAST and placebo groups for each of the 26 gray matter ROIs and 19 white matter tracts. If there was a significant BL difference for any of the MLM derived brain connectivity measures, then this region or tract was not included in computation of correlations.

Pearson correlation coefficients were computed to reveal neural connectivity changes associated with neurobehavioral gains for the FAST and placebo interventions. For all brain connectivity measures not significantly different between FAST and placebo groups at BL, the MLM-derived rsFC and FA measures of change were used to compute correlations with MLM-derived DOCS-25 Total, DOCS-25 Auditory-Language and CNC measures of neurobehavioral change.

In the effort to identify robust relationships, significance of correlations between changes in neural connectivity and neurobehavioral gains as well as between resting state networks were tested. Significance was tested using *t*-tests for Pearson correlations and *t*-tests for Fisher *z* transformed values, respectively. We examined significance of correlations within and between FAST and placebo groups.

Data Analyses: Verifying the Merits of a Positive Correlation for the FAST Intervention

When a significant positive correlation for FAST participants was identified using above procedures, then this relationship was verified by recomputing correlations using adjusted metrics of neural connectivity, specifically FA. The FA metrics, for specific fiber tracts, were adjusted by standardizing the amount of substrate (i.e., white fiber tract length) while controlling for individual brain size (i.e., normalization) (also see **Supplement C** for additional details on computations and verifications).

Standardizing tract length, by using fixed length for a tract across subjects, is important as this accounts for injury heterogeneity. One subject may, for example, have substantially more intact fiber tract (i.e., longer discernible tracts in DTI) than another and, considering the small sample size, this could overestimate or underestimate the correlation with neurobehavioral gains. To avoid overestimation of the correlation, we examined the lengths of a given fiber tract across all subjects and then used the smallest length to define the tract length for all subjects. Using a fixed length across all subjects means that, for some subjects, partial volumes were used. However, the approach of using a fixed length, defined by the smallest length across subjects, neutralizes the possibility of overestimating the correlation. Notably, this approach could underestimate the correlation, but given the objective of verifying a positive correlation, we chose to avoid overestimation. Considering that each subject also has his/her own unique brain size, the fixed tract length was then normalized for each subject according to the normal persons' brain size (MNI template). In summary, for any fiber tract with changes in structural connectivity found to be significantly and positively correlated with neurobehavioral gains for FAST participants, we conducted a verification analysis. This verification involved adjusting the relevant FA measures to be (a) standardized by amount of substrate across subjects and (b) normalized by brain size according to MNI brain volume. To verify the finding of a positive correlation, we then re-estimated change in brain connectivity measures from BL to EP by using the adjusted FA measures in the MLM described above. These MLM re-estimated FA measures were then used to recompute Pearson correlations between FA change and neurobehavioral change. Correlations remaining positive were identified as robust relationships warranting further examination in future research.

RESULTS

Examinations of the precision of MLM estimated measures of brain connectivity metrics indicate very small MAE and MARE and no significant differences between the actual and estimated values ($p = 0.999$) (also see **Table SC1** in Supplement C). Accordingly, all neural connectivity results are based on the MLM estimated z and FA measures. For reference, results based on raw neural connectivity values are provided in **Tables SD4,5** in Supplement D.

Representativeness of Pilot Study Participants

At BL, the RCT participants included in the imaging subgroup ($n = 8$) and the RCT participants excluded from the pilot study ($n = 7$) did not differ according to demographic factors (e.g., age, time post TBI), clinical states (e.g., VS, MCS), prognostic factors (e.g., comorbidities, time post-injury, lesion location, type), usual-care services (e.g., pharmacological, therapeutic content), or by DOCS-25-total and CNC measures of neurobehavioral function (all $p > 0.05$; see **Table SE1** in Supplement E, columns A–C). The only difference between RCT participants excluded and included in the pilot study was cause of injury. The majority of excluded participants (5/7) and one included participant (1/8) were injured in automobile accidents ($p = 0.01$). The MLM-derived linear trend lines indicated that the RCT participants included in the pilot study were similar to the excluded participants according to neurobehavioral function (all $p > 0.05$).

Pilot Study Participants: Imaging Subgroup Composition

The imaging subgroup ($n = 8$) comprised largely men (88%) who incurred a TBI at an average age of 40 years and who, at time of RCT enrollment, had remained in states of DoC an average of 78 days (**Table 1**, also see **Supplement E**). At BL, the majority (62%) of participants presented with behavioral characteristics consistent with MCS. At treatment EP, 25% of the subgroup remained in VS, 25% either progressed to or remained in MCS, and 50% emerged from MCS.

The pilot study participants who received the FAST ($n = 4$) vs. those receiving the placebo ($n = 4$) intervention did not differ at BL according to demographics, prognostic factors, and usual care (all $p > 0.05$; see **Table SE1** in Supplement E, columns D–F). The groups also did not differ ($p = 0.46$) according to time between injury and study BL (FAST: mean = 117.0, SD = 56.2; median = 111, range = 59–187) (placebo: mean = 91.0, SD = 34.5; median = 88; range = 52–136). The FAST and placebo participants also did not significantly differ by type and location of brain lesions, but placebo participants had a slightly higher number of contused brain regions and total number of lesions.

BL mean rsFC strength within each neural network was similar between FAST and placebo participants (**Table 2**, column G). For rsFC strength between-networks at BL, the FAST participants had significantly (ordered $p = 0.04$) weaker correlation ($z = 0.62$) between AN–DMN relative to participants receiving the placebo ($z = 0.74$).

BL FA values between the FAST and placebo participants (see **Table SF5** in Supplement F) did not significantly differ for 18 of the 19 tracts examined (all $p > 0.05$). As the FA of the left inferior fronto-occipital fasciculus (IFOF) was significantly higher for the FAST participants (mean FA = 0.37, SD = 0.01; placebo mean FA = 0.35, SD = 0.01; $p = 0.03$), the FA for the left IFOF was not considered in final interpretations.

FAST Facilitated Changes in Functional Connectivity

Within-Group Differences

The FAST participants had three significant within-network changes in rsFC strength. Specifically, mean z values decreased from BL to EP for the AN, LN, and SN (**Table 2**, column C, EP–BL). The FAST participants also had two significant between-network changes with rsFC strength: decreasing for AN-LN and increasing for DMN-SN (**Table 2**, column C).

There were no significant within-network rsFC strength changes for the placebo participants (**Table 2**, column F). There were, however, significant rsFC changes between-networks: rsFC of AN–DMN decreased and AN-LN increased.

TABLE 2 | Resting state functional connectivity within and between networks: pilot study imaging subgroup FAST and placebo participants.

	FAST ($F_n = 4$)			Placebo ($PL_n = 4$)			FAST vs. Placebo	
	A	B	C	D	E	F	G	H
	Mean correlations (*Z* values*)		Change (EP—BL)	Mean correlations (*Z* values*)		Change (EP—BL)	Baseline (BL)	Endpoint (EP)
Networks	Baseline	Endpoint	Ordered *p*	Baseline	Endpoint	Ordered *p*	Ordered *p*	Ordered *p*
AN	0.32	0.29	**<0.01**	0.27	0.27	0.40	0.16	0.29
DMN	0.33	0.28	**0.06**	0.29	0.29	0.53	0.23	0.36
LN	0.52	0.41	**<0.01**	0.44	0.42	0.33	0.34	0.47
SN	0.47	0.30	**<0.01**	0.42	0.41	0.40	0.17	**0.04 (*F* < PL)**
AN–DMN	0.62	0.67	0.53	0.74	0.67	**<0.01**	**0.04 (*F* < PL)**	0.49
AN–LN	0.53	0.29	**<0.01**	0.44	0.53	**<0.01**	0.14	**0.0 (*F* < PL)**
AN–SN	0.04	0.31	0.13	0.32	0.30	0.33	0.23	0.47
DMN–LN	0.67	0.65	0.13	0.54	0.56	0.53	0.13	0.34
DMN–SN	0.45	0.55	**<0.01**	0.47	0.43	0.27	0.55	0.27
LN–SN	0.47	0.76	0.13	0.74	0.68	0.27	**0.08**	0.35

**z, mixed-effects linear model estimates; ordered p, independent-sample permuted t-tests conducted with mean estimated z values; Black shaded cells = ordered p ≤ 0.05 where significance testing accounted for multiple comparisons using FDR q = 0.20; Gray shaded cells = ordered p > 0.05 and < 0.10; F > or < PL: FAST group mean is > or < than placebo group mean at; AN, attention network; DMN, default mode network; FAST, familiar auditory sensory training; LN, language network; SN, salience network.*

Between-Group Differences

At treatment EP, the placebo participants had significantly stronger rsFC within SN. The mean *z* value for the FAST group declined from 0.47 to 0.30 but remained stable for the placebo group (0.42 and 0.41). The placebo participants also had significantly stronger rsFC between AN-LN (**Table 2**, column H) with mean *z* for AN-LN increasing for the placebo group and decreasing for the FAST group.

FAST Facilitated Changes in Structural Connectivity

Within-Group Differences

FAST participants had significant increases in unadjusted FA for three tracts: the right superior longitudinal fasciculus (SLF), the right arcuate fasciculus (AF), and the right uncinate fasciculus (UF) (**Figure 2C**; also see **Table SF1** in Supplement F). All three tracts decreased bilaterally for the placebo participants, with decreases in the right AF reaching statistical significance.

The placebo participants had significant FA declines of the splenium and body of the corpus callosum, whereas FAST participants had non-significant declines in FA for these same tracts (**Figure 2B**).

Between-Group Differences

There were significant differences between FAST and placebo participants in the unadjusted FA at EP, for the left and right inferior longitudinal fasciculus (ILF) (**Figure 2A**). This finding was due to a decrease within the ILF for the placebo participants and an increase for the FAST participants. Both groups demonstrated an increase of the unadjusted FA of the right superior frontal-occipital fasciculus (SFOF); the FAST participants increased more than the placebo participants.

Relationships Between Changes in Connectivity and Neurobehavioral Gains

The MLM-derived measures of change in neurobehavioral function and neural connectivity (rsFC and DTI) were used to reveal FAST treatment-related changes in neural connectivity associated with neurobehavioral recovery. For rsFC, there were no significant associations between indices of neurobehavioral change within any rsFC network or between any network–network pair (see **Tables SF2, 3** in Supplement F). For DTI, however, the FAST participants' changes in mean FA of the left ILF were significantly and positively correlated with the DOCS-25 Auditory-Language measure ($p = 0.02$). For placebo participants, increased FA of right UF was also significantly and positively correlated to improving arousal and awareness as measured by the CNC ($p = 0.03$) (also see **Figure SF1** in Supplement F).

Verifying the Relationship Between ILF Change and FAST-Related Neurobehavioral Gains

The positive correlation for FAST participants between the left ILF and the DOCS Auditory-Language gains was verified by recomputing Pearson correlations using the adjusted FA for the left ILF (**Figure 3**, i and ii). Although not significant ($p > 0.05$), the correlation using the adjusted FA measures remained positive and moderately strong ($r = 0.59$) for FAST participants (**Figure 3**, iii). For the placebo participants, however, the correlations changed from positive to negative ($r = -0.58$).

As depicted in **Figure 3** (panel iii and Data table), three of the four FAST participants and all four of the placebo participants made meaningful DOCS-25 Auditory-Language gains. Specifically, meaningful gains are indicated when the

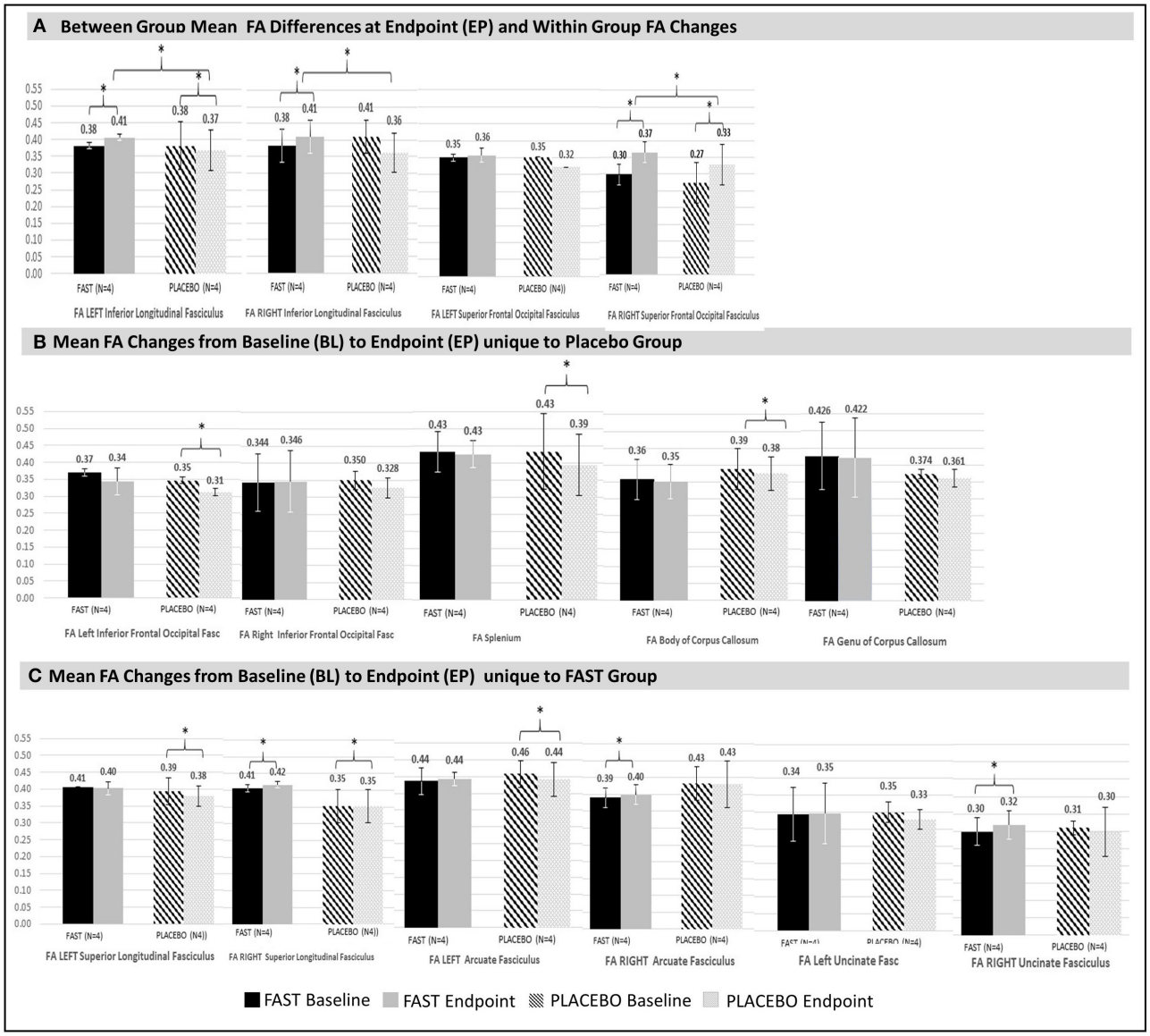
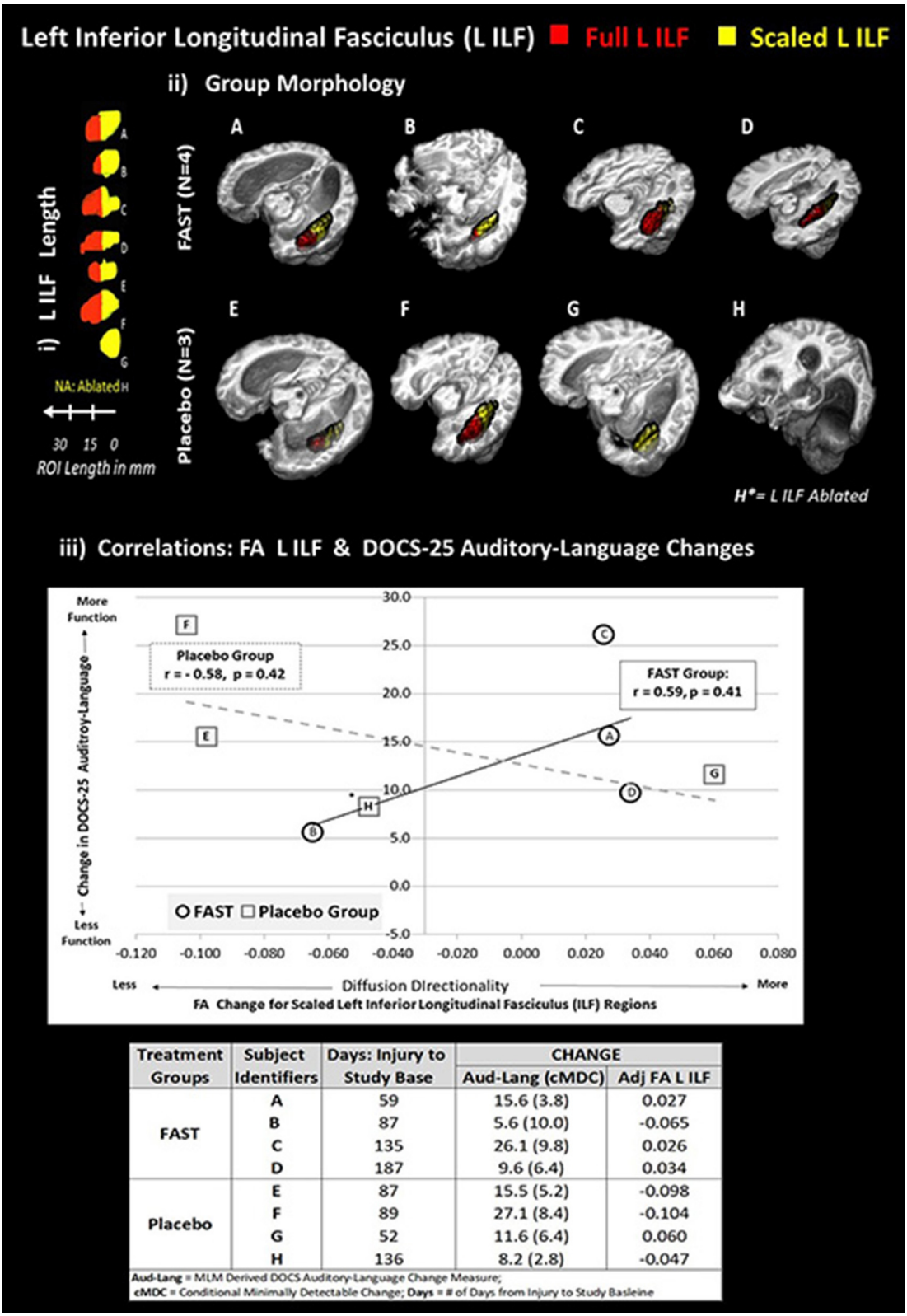


FIGURE 2 | Significant changes in structural connectivity between and within groups. Significant (denoted with * with all $p < 0.00$) mean FA differences between and within groups at endpoint are illustrated. For comparison purposes, non-significant FA values for contralateral tracts are also provided. Vertical bars in black and white indicate standard deviations. FAST group is denoted with solid black (baseline) and gray (endpoint) bars. Placebo group is denoted with patterned black (baseline) and patterned gray (endpoint) bars. **(A)** Between- and within-groups: Between-group mean FA endpoint differences and within-group FA changes from baseline to endpoint. FAST group's FAs for the left and right ILF at endpoint are greater than same measures for Placebo group. Change in FA of the right SFOF increases for both groups, but the FAST group increases more than the placebo group. **(B)** Placebo group: Mean FA changes from baseline to endpoint within tracts unique to the placebo group were all decreases. Decreases occurred in the right AF, splenium, and body of corpus callosum. The left IFOF also significantly declined, but at baseline this was significantly lower for the placebo group. Thus, the left IFOF finding is not considered for interpretation. Although there were no significant changes for the genu, this tract is shown to facilitate evaluation of all colossal fiber tracts. **(C)** FAST group: Mean FA changes from baseline to endpoint that are unique for the FAST group occurred within three right hemisphere tracts: right SLF, the right arcuate fasciculus (AF), and the right uncinate fasciculus (UF).

participant's neurobehavioral gains exceed their measurement error defined as the conditional minimally detectable change (65) **Figure 3** also indicates that the adjusted FA measures for the left ILF improved for the same three FAST participants (A, C, and D), but decreased for three of the four placebo participants. More specifically, these three FAST participants made gains in the adjusted FA of the left ILF and the DOCS Auditory-Language measures. Participant A was earliest after injury (59 days) and made more gains than the other two participants C and D who were studied 135 and 187 days post-injury, respectively. In contrast, the only placebo participant making gains in both DOCS auditory-language skills and



Treatment Groups	Subject Identifiers	Days: Injury to Study Base	CHANGE	
			Aud-Lang (cMDC)	Adj FA L ILF
FAST	A	59	15.6 (3.8)	0.027
	B	87	5.6 (10.0)	-0.065
	C	135	26.1 (9.8)	0.026
	D	187	9.6 (6.4)	0.034
Placebo	E	87	15.5 (5.2)	-0.098
	F	89	27.1 (8.4)	-0.104
	G	52	11.6 (6.4)	0.060
	H	136	8.2 (2.8)	-0.047

Aud-Lang = MLM Derived DOCS Auditory-Language Change Measure;
cMDC = Conditional Minimally Detectable Change; **Days** = # of Days from Injury to Study Basleine

FIGURE 3 | Each subject's left ILF by group and correlation with DOCS-25 auditory-language measures. The top half of figure illustrates the full left ILF, depicted in red and adjusted left ILF, depicted in yellow. Panel i shows the length in millimeters (mm). Panel ii demonstrates the group-level morphology in native space. To standardize the amount of substrate, relative brain size, and location of the ILF comparisons, an adjusted portion of each subject's left ILF was used to compare FA

(Continued)

FIGURE 3 | Continued
values. The following formula was applied:
Adjusted left ILF = [(subject's skull length a–p)/(MNI skull length a–p) * shortest ILF].
1) All ILFs were limited to shortest ILF in the in anterior to posterior (a–p) plane (i.e., G had the shortest ILF at 14 voxels with 1-mm^3 resolution).
2) The 14-voxel length was then scaled for each subject based on the ratio of their maximum skull length over the maximum skull length of the MNI152 brain (both in the a–p plane).
3) To extract comparable FA values, the adjusted left ILF length was measured and masked from the posterior pole of the ILF in the a–p plane.
Plot on bottom half of figure panel iii indicates that the scaled FA left ILF values are, for the FAST group, positively correlated with improved DOCS-25 Auditory-Language abilities. The plot depicts trend lines by group for change between baseline and endpoint in scaled left ILF FA and DOCS-25 Auditory-Language measures reported for each subject. *Subject H is missing the left ILF, and this FA value was estimated using imputation methods. Each subject's alphabetic label corresponds with panels i and ii and the change indices provided in the table in bottom of panel iii.

the adjusted FA of left ILF was participant G, who, of all the placebo participants, was studied the earliest after injury (52 days).

DISCUSSION

In our pilot study of persons with DoC due to TBI, we examined longitudinal data for an imaging subgroup derived from a double-blind placebo-controlled RCT (33). We discovered relationships between the FAST intervention and changes in structural and functional neural connectivity, one of which was positively associated with neurobehavioral gains. To identify FAST-related relationships warranting further examination in future research, we used significance testing to filter the revealed relationships, and we verified the positive correlation. For a patient population with many scientific challenges, this report demonstrates a systematic approach to rigorously explicating these relationships. This scientific approach provides a basis for identifying the unique contributions of specific interventions to recovery, thereby providing a foundation for developing complementary treatments for a patient population with limited treatment options. If the FAST intervention is examined further, and findings are replicated in future research, then these findings could delineate the unique contributions of the FAST intervention to recovery and, ultimately, inform development of targeted treatment strategies to improve brain and behavioral function for persons with DoC after TBI.

FAST Intervention: Structural Connectivity and Auditory-Language Skills

A key finding that merits replication in future research is the positive association between the FA of the left ILF and the DOCS-25 Auditory-Language measures. Further investigation is merited, in part, because the positive direction of the association for the FAST group was verified by recomputing the correlations using an adjusted FA. Considering that adjusting the FA involved using, across all subjects, the smallest length of the left ILF to define a fixed tract length for all subjects, this approach means that we used partial volumes for the other participants, thereby underestimating the strength of the correlation. As the objective was to verify the presence of a robust relationship, this approach was selected because it avoided overestimation. Additional evidence indicating that this relationship is robust includes the findings that the FAST group had significantly higher mean FA of the left IL at treatment EP (**Figure 2A**). Furthermore, findings suggest that time post-injury or the potential for change in structural connectivity due to innate recovery did not prohibit gains in the FA of the left ILF. Descriptive findings indicate that the time post-injury was balanced between the FAST and placebo groups. Also, three of the four FAST participants and only one placebo participant made gains in both the auditory-language skills and the FA of Left ILF. Notably, this placebo participant was the earliest after injury, whereas the FAST participants ranged from 59 to 187 days after injury. Considering that (a) both the FAST and placebo groups improved in DOCS-25 auditory-language skills, (b) the adjusted FA of the left ILF increased for the majority of the FAST participants but decreased for the majority of the placebo participants, and (c) the collective evidence suggests that time post-injury did not prohibit gains in FA of the left ILF, future research should focus on understanding the relationship between the FAST and left ILF, particularly the role of the left ILF relative to time post-injury.

We speculate that additional future examinations could indicate that the FAST intervention facilitated connectivity changes and/or prevented degradation within the left ILF. Based on our RCT findings (33) and a previous case report, (66) we also speculate that these findings will be present in the acute through the chronic stages of recovery but that the effects will be more pronounced earlier after injury.

Future research to further advance the understanding of likelihood of these FAST intervention effects and/or explicating other effects of the FAST within the left ILF is particularly valuable when considering the involvement of the left ILF in language function. The left ILF is a long intrahemispheric association pathway connecting the anterior temporal lobe to the occipital lobe (67, 68) intersecting with the posterior segments of the AF, the UF, and the IFOF. Importantly, these three tracts are all thought to serve as components of the ventral language pathway, and each is implicated in the rehearsal component of language recovery (69, 70). The IFOF is also thought to play a particularly important role in semantic processing. Because the unadjusted FA of the Left IFOF was significantly higher for the FAST participants at BL, we did not examine its role. Future studies should, however, examine the role of the left IFOF relative to the left ILF. Further consideration of (i) the role of the ventral language pathway in providing repeated and recurring opportunities for learning, particularly when there are constraints on working memory, (71) and that (ii) the FAST intervention provides repeated exposure to language-based episodic stories also highlight the need to identify

the unique and/or synergistic contributions of the features of the autobiographical auditory-linguistic stimuli (i.e., language, speech patterns, autobiographical content, emotions, familiar voices). More specifically, the FAST RCT was not designed to delineate the roles and contributions of each feature alone vs. together or to explicate the amount of repetition necessary (i.e., dose).

A final consideration regarding the merits of future examinations of this complex relationship is the potential impact of replicating an association between the left ILF and DOCS-25 auditory-language skills. If replicated, then this association would indicate that the FAST intervention, consisting of autobiographical auditory-linguistic stimuli, provides targeted stimulation to specific neural networks resulting in alterations of structural connectivity, and importantly, further explication would advance understanding of the role of these changes in supporting auditory-language skills. Given the importance of the left ILF to language and recovery of consciousness, (72) our findings of increased left ILF connectivity with provision of the FAST intervention as well as the potential clinical impact, the presence and nature of the complex relationship among the FAST intervention, the left LF and DOCS-25 auditory-language skills merits additional examination in future research.

FAST Intervention: Structural Connectivity of Right Hemisphere Language Homologs

An additional relationship identified as meriting further investigation is the finding that only the FAST group had increased structural connectivity of the right SLF and right AF without any neurobehavioral associations. If this finding is replicated in future research, we theorize that this would indicate that the FAST intervention facilitates redirection of resources to right hemisphere language homologs and that these changes may not be sufficient to support neurobehavioral recovery, and/or these changes precede neurobehavioral recovery. This hypothesis is plausible when considering that the right SLF, right AF, and left ILF are long-range association fiber tracts with overlapping cortical projections supporting multiple complex functions via polymodal brain hubs (68, 73–78). Although there is evidence that DoC patients have an impaired ability for a brain hub to connect with spatially distant hubs, (79) these long-range association fiber tracts are capable of responding to multiple modalities. Also congruent with this hypothesis is prior research showing that function within these polymodal areas recovers after patients regain conscious behaviors (80). The idea of FAST treatment–induced resource redistribution is similar to previous suggestions of resource distribution related to the provision of usual care (81). The plausibility of FAST-induced redistribution is bolstered by a growing body of evidence indicating that the right hemisphere plays important roles in facilitating language functions and recovery after brain injury (33, 69, 82–84) and by our previously reported findings that FAST RCT participants had increased neural activation in the right hemisphere homologs of language processing in response to a non-familiar voice reading aloud a novel story (33).

We considered the possibility that, rather than redistribution, the FAST intervention engaged the right SLF and AF in a manner similar to the relateralization of language processing to intact homotopic right-hemisphere regions observed in language recovery after left hemisphere stroke. (27, 28, 85–87). However, the literature regarding right hemisphere compensatory functions is mixed with some findings, suggesting that right hemisphere engagement is pathological (88–91). When considering the findings of increased connectivity of the right SLF and AF in the context of increased FA in the left ILF, we posit that the injured brain's response to the autobiographical auditory-linguistic stimuli could include engagement of targeted networks that serve to redirect brain resources rather than a clear relateralization effect seen in language recovery with aphasia after stroke (83). The absence of correlations between the FA increases in right SLF and AF and neurobehavioral gains highlights the need for future mechanistic research investigating the idea of redistribution as well as to determine the necessary and sufficient levels of connectivity within the right SLF and AF to support neurobehavioral gains.

Structural Connectivity Changes Relative to Usual Care and Endogenous Recovery

Both FAST and placebo groups had significant increases in FA of the right SFOF from BL to EP. Because both groups received usual care, this finding suggests that usual care and/or endogenous recovery contributed to the increases in structural connectivity of this fiber tract. Notably, the FA increased more for the FAST group such that the FA was significantly higher at EP compared to the placebo group (**Figure 2A**). Replication of this finding in future research would suggest that usual care and/or endogenous recovery engage the right SFOF and that there is more engagement when the FAST intervention is paired with usual care.

Functional Connectivity Changes for FAST and Placebo

For the FAST group, rsFC decreased within the AN, LN, and the SN and increased between the SN and DMN (**Table 2**). Given the roles of each network and that the FAST stimuli are intended to target these networks, rsFC changes within and between these networks were expected. The direction of several of the rsFC changes, however, was unexpected. The rsFC of the AN-LN, for example, decreased in the FAST group and increased for the placebo group (**Table 2**). We expected the opposite, in part, because the linguistic components of the FAST stories are designed with the intention of engaging the LN (69). Furthermore, the AN supports the ability to volitionally (i.e., dorsal AN) attend to a task and to reflexively (i.e., ventral AN) attend to or detect a stimulus (92–94). These unexpected findings, in terms of direction of changes, are noteworthy because the degree of hyperconnectivity and hypoconnectivity in DoC remains poorly understood (8, 22–26) and warrants further research to inform therapeutic strategies.

Given the features of the stimuli used in the FAST intervention and the roles of each rsFC network, we were also surprised that changes in rsFC in the FAST group were not correlated to neurobehavioral recovery. The SN, for example, is thought to be targeted with the personalized auditory-linguistic stimuli (37) because this network supports the ability to orient toward salient emotional stimuli, (95) consciously perceive stimuli, (96–100) and detect and attend to stimuli (92–94). After TBI, coordinated SN–DMN interaction is also impaired, (101) and the discordance of interactions impedes efficient attention switching from internal processes to salient external stimuli. The surprising lack of associations may be related to the small sample size, which also precluded an examination of paired regions.

In lieu of interpreting these unexpected findings based on a small sample, we highlight them here to note the need to reexamine associations between rsFC changes and neurobehavioral recovery with a larger sample. The relationship between the FAST and changes in rsFC of these networks is an important area for further research, in part, because of knowledge that overt participation in a task requiring focused attention is necessary to activate the AN, LN, and SN. Thus, our findings of FAST intervention–related rsFC changes represent the first reported evidence that these broad neural networks can be engaged with a sensory treatment that does not require overt task participation (102–104). If future research findings are consistent, then clinicians will be able to provide treatments targeting broad neural networks important to recovery.

Limitations

The robust methods employed in this pilot study, combined with use of data from a double-blind placebo-controlled RCT, provide confidence in the reported findings; however, the small sample size places limits on generalizability to the larger DoC population. The verified association between the adjusted FA of the left ILF and DOCS-25 Auditory-Language measures, for example, is based on four subjects per group. While this is a potentially important finding, it should be replicated in future research. The purpose of the study was to identify relationships meriting further investigation, and accordingly, we used FDR threshold of 0.20 for multiple comparisons. Although we chose this liberal threshold to avoid obscuring relationships that could provide insights for future research, this threshold means that for every 5 discoveries, there would be one false discovery and four true discoveries (105).

The imaging data, particularly DTI, were collected prior to the development of methods to correct for geometrical distortions (e.g., field maps and reversed phase encode). We did, however, correct for geometric distortions in our diffusion processing pipeline. First, the b-0 image was non-linearly transformed to the 3D anatomic scan. Next, each gradient direction was individually transformed to the $b = 0$ image through non-linear warping. This approach matches the diffusion data to the anatomic data reasonably well. Furthermore, the results are not in regions that might exhibit higher levels of distortion such as the orbital frontal cortex or brainstem.

Within-subject variability unique to rsFC data may not have been sufficiently minimized (106–109). The uniqueness of the within-subject variability associated with rsFC together with the small sample size for between-group comparisons indicates a need for replication of the rsFC findings or implementation of a different design that allows for examination of associations with neurobehavioral changes. The rsFC analyses and findings provide a framework that can be applied when replicating this study.

All analyses were conducted using a framework of *a priori* selected networks. While this approach is critical to advancing knowledge in unchartered areas of science, it also means that some changes in connectivity may have gone undetected (110). The *a priori* approach also limits the ability to identify connectivity between-network nodes (e.g., right SLF) with other polymodal hubs, which in turn engage other networks.

For our *a priori* approach with rsFC, we used established resting state networks consisting of gray matter regions with established roles. This approach allows us to interpret our findings relative to other studies, but this also means that the auditory-language network is a particularly simplistic representation of the language connectome (69, 111). For the unadjusted FA values, we also drew full white matter tracts for each subject because we sought to explore the function of the entire tract. This approach of averaging the entire white fiber tract assumes equipotentiality within each white matter tract. As a result, we may not have detected more subtle changes in structural connectivity.

Future Directions

Future research examining the relationships identified above as well as potential connections between the left ILF, right AF, and right SLF with other networks can provide insights about how to target treatments to support recovery of auditory-language skills. Determining the unique contributions of any treatment to neurobehavioral recovery requires the development of methods to address the many challenges with DoC research. Thus, the methods employed in our scientific approach must be replicated and refined to identify the best approach for modeling these complex data. Future research also needs to systematically define clinically meaningful changes in functional and structural connectivity for people with DoC that can be used to examine efficacy of targeted treatments in clinical trials. The replication and further examination of these important relationships, refinement of the methods we employed, and advancing knowledge of clinically meaningful changes in neural connectivity will further enable determination of the unique contributions of the FAST and other interventions and inform development of targeted treatments.

The state of DoC science informed our network-level approach to identify functional and structural connectivity changes and to examine the relationship between these changes and neurobehavioral gains. This method, though, is not a direct causal approach and precludes definitive determinations of mechanisms. Further research examining the role or contributions of alternate white matter pathways to neurobehavioral recovery from DoC after TBI is needed to definitively identify viable therapeutic targets.

Prior research has demonstrated that language gains are likely to be related to white matter microstructure and association fibers that link gray matter regions to enable the cross-modal integration required for higher-order complex behaviors (112). Consistent with this premise is the reported finding of no correlations between rsFC and neurobehavioral changes and presence of positive correlation between increasing structural connectivity and neurobehavioral gains. This consistency between previous research and our findings identifies a need for research examining the convergence and divergence of rsFC and structural connectivity during neurobehavioral recovery using a direct causal approach (113).

To develop effective neuromodulatory interventions, studies similar to ongoing research in stroke (21) are needed to advance knowledge of the relationship of hyperconnectivity and hypoconnectivity with neurobehavioral recovery from DoC. The unexpected findings of direction of rsFC changes highlight the need for research determining the degree that hyperconnectivity and hypoconnectivity in DoC is pathological (8, 22–26). Investigating this construct is important for informing development of treatments targeting attenuation or excitation of functional connections.

The incongruences in some of the reported findings highlight the challenges involved in characterizing and interpreting recovery and/or reconfiguration of network connectivity in a severely damaged system, particularly when considering novel patterns of activation/deactivation related to a specific treatment. The incongruences highlight the importance of considering how a damaged network may fluctuate over time. It is plausible that treatment-induced connectivity changes cause a cascade of fluctuations in activations and deactivations that unfold over different time scales as each network attempts to repair itself. We have addressed this challenge by using longitudinal data derived from a placebo-controlled RCT, thereby enabling a comparison of the FAST and placebo groups. Future work, however, would benefit from taking repeated measures at more than two timepoints during provision of the intervention to assess within-subject variability. This challenge highlights the need for future research on how the damaged brain repairs and recovers as well as how treatment alters this recovery. Higher-order diffusion models that can account for multiple fibers in a single voxel are also needed to advance this knowledge.

CONCLUSIONS

For persons with DoC after TBI, our findings collectively represent an important first step toward understanding the unique contributions of the FAST intervention, which is a simple passive sensory treatment consisting of autobiographical auditory-linguistic stimuli that are provided with the intention of engaging specific and broad neural networks known to be important to recovery. The findings warranting further investigation and replication in future research are those suggesting that the FAST may induce structural and functional connectivity changes, some of which associated with auditory-language gains.

The reported findings make unique and clinically meaningful contributions to the field of neurorehabilitation while expanding on previously published findings demonstrating that the FAST intervention is related to improved awareness and language skills. While there are reports of increased task activation after recovery to passive sensory stimulation in differing gray matter regions, (33, 81, 114) to our knowledge there has never been a placebo-controlled RCT-based report of resting state functional and structural connectivity changes related to provision of a passive yet targeted sensory stimulation treatment in DoC patients. Generalizability of our findings to all patients with DoC is, nonetheless, premature. If our findings are supported in future studies, however, then this low-cost, effective treatment can be readily modified and clinically implemented. For a patient population with limited treatment options, this line of research could have important therapeutic implications.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hines VA, Richmond VA and Northwestern Unviersity's IRBs. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception or design of the work, or the acquisition, analysis or interpretation of data for the work, and drafted the submitted work and/or revised it critically for important intellectual content, and also provide approval for publication of the content and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.01027/full#supplementary-material

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Deletion of MicroRNA-144/451 Cluster Aggravated Brain Injury in Intracerebral Hemorrhage Mice by Targeting 14-3-3ζ

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This study aims at evaluating the importance and its underlying mechanism of the cluster of microRNA-144/451 (miR-144/451) in the models with intracerebral hemorrhage (ICH). A model of collagenase-induced mice with ICH and a model of mice with simple miR-144/451 gene knockout (KO) were used in this study. Neurodeficits and the water content of the brain of the mice in each group were detected 3 days after collagenase injection. The secretion of proinflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β), as well as certain biomarkers of oxidative stress, was determined in this study. The results revealed that the expression of miR-451 significantly decreased in the mice with ICH, whereas miR-144 showed no significant changes. KO of the cluster of miR-144/451 exacerbated the neurological deficits and brain edema in the mice with ICH. Further analyses demonstrated that the KO of the cluster of miR-144/451 significantly promoted the secretion of TNF-α and IL-1β and the oxidative stress in the perihematomal region of the mice with ICH. In addition, the miR-144/451's depletion inhibited the regulatory axis' activities of miR-451-14-3-3ζ-FoxO3 in the mice with ICH. In conclusion, these data demonstrated that miR-144/451 might protect the mice with ICH against neuroinflammation and oxidative stress by targeting the pathway of miR-451-14-3-3ζ-FoxO3.

Keywords: microRNA-451, intracerebral hemorrhage (ICH), inflammation, 14-3-3ζ, mice

INTRODUCTION

As a devastating stroke subtype, intracerebral hemorrhage (ICH) is related to poor prognosis and high disability (1). Serious sequela of survivors and the lack of effective clinical treatments bring health burdens to the patients with ICH and their families (2). Currently, many treatments need to be explored in preclinical and clinical researches of ICH, but there is no effective treatment (3). Thus, it has been suggested that the development of novel and effective therapeutic treatments for ICH remains an important area of preclinical researches.

The pathogenic processes of ICH include the primary and secondary brain injuries. The primary brain injuries stem from rapid hematoma and then develop various biological effects including oxidative stress and neuroinflammation, leading to secondary brain injuries. Neuroinflammation cascade, involving microglia activation, secretion of proinflammatory cytokines, and oxidative stress, accelerates neuronal death and edema exacerbation after ICH (4, 5). Therefore, the identification of mechanisms of inflammation following ICH may provide promising strategies for brain injuries and poor prognosis.

As non-coding RNAs, microRNAs (miRNAs) inhibit the posttranscription of the target genes through binding to their 3′ untranslated region (6–8). miRNAs have been proposed to be novel biomarkers and regulatory molecules in the diagnosis and treatment of ICH (9). Increasing studies show that miRNAs are involved in the pathogenesis and development of ICH in preclinical researches (10, 11). For example, it is found that miR-126-3p mimic administration significantly alleviated the neurological deficits of the mice with ICH and inhibited BBB disruption and cerebral edema by targeting the pathway of PIK3R2-mediated PI3K/Akt in the perihematomal area (10). Qu Xin et al. reported that miR-146a mimic injection obviously improved the motor function and alleviated the brain edema, suppressed the secretion of proinflammatory cytokines tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β), and suppressed the oxidative stress around hematomas of the mice with ICH (11).

miR-144 and miR-451 (miR-144/451) with high conversion in different species were processed from a single RNA precursor transcript and abundantly expressed in erythrocyte precursors (12). miR-144/451 facilitated terminal maturation of erythrocyte precursors and protected from oxidant stress through down-regulating 14-3-3ζ in erythroid cells (13). Knockdown of miR-144/451 triggers the risk of lung, gastric carcinoma, and bladder cancer, suggesting the cluster as a potential tumor suppressor (14–16). It has been found that miR-451 could suppress the microglia-mediated inflammation in chronic inflammatory pain by targeting TLR4 (17). Liu et al. showed that miR451 could protect neurons against oxidative stress in an oxygen and glucose deprivation/reoxygenation (OGD/R) cell model through inhibiting its target protein-CUGBP Elav-like family member 2(CELF2) (18). Fu et al. reported a negative association between miR-451 levels in blood of ischemic stroke patients and National Institutes of Health Stroke Scale scores and infarct volume (19). Little is known on the behavior of miR-451 in the development of post-ICH complications, namely, edema and neuroinflammation.

Thus, the work reported herein aims to address the effects of the cluster of miR-144/451 on behavioral deficits, neuroinflammation, and oxidative stress around hematomas in mice model with ICH. This work first evaluated the importance of the cluster of miR-144/451 during the occurrence of ICH using the mice with miR-144/451 gene knockout (KO).

MATERIALS/METHODS

Animals/the Establishment of Model With ICH

Animals

Gene KO mice lacking miR-144/451 cluster (referred to as KO) were defective in miR-144/451 expression in all kinds of tissue as previously described (13). The KO mice were obtained from University of Pennsylvania and kept in a standard pathogen-free and quiet room. Wild-type mice with same background (C57BL/6J) were used for the control. The animals were divided into four groups ($n = 15$) randomly: a sham, an ICH, an ICH + miR-144/451 KO (ICH + KO), and a sham + miR-144/451 KO group. The protocols used in this study were with the approval of the Institutional Animal Care and Use Committee and the Animal Ethics Committee of Yangzhou University [SYXK (Su) IACUC 2017-0045]. These animals were all kept in a room under a 12-h light/dark cycle at 23°C ± 1°C with free access to water/foods.

The Establishment of the Model of ICH

These animals were anesthetized by intraperitoneal administration of 4% chloral hydrate and then fixed onto a stereotaxic frame. We then located the right caudate putamen using the stereotactic coordinates (coordinates related to bregma, anteroposterior (AP), mediolateral (ML), and dorsoventral (DV), are 0.2, 3.7, and 3.8 mm, respectively) according to the previous description (20). Type IV collagenase (0.05 U) (Sigma–Aldrich Co., St. Louis, MO, USA) was injected using a syringe. The needle had been kept in the brain for 5 min additionally to prevent back-leakage. The mice in the sham group and KO group were injected with the same volume of normal saline.

Measurements of Neurological Outcome

During the neurological function tests, all of the animals were kept from any stress, and their well-being was monitored. All of the behavioral training and tests were carried out in a quiet room at a fixed time point everyday by at least two experimenters blinded to the behavioral test.

Cylinder Test

The cylinder test was performed by an examiner blinded to the experiment for the measurement of spontaneous forelimb use. The mice ($n = 9$ per group) had been put in a transparent cylinder and videotaped for 5 min. A forelimb was placed on the wall for the first time, and the later movements along the wall were counted. The percentage of the affected limb use was calculated.

Rotarod Test

Motor coordination of the mice was investigated using the rotarod test. In brief, all animals ($n = 9$ per group) had been trained by an examiner blinded to the experiment, at the speed of 4–30 revolutions/min (rpm) for 5 min, three times per day, for 3 days before the induction of ICH. The mice that fell off the rod were put back with minimal disturbance. The mice that had not achieved stable performances after training were kicked from the test. After the operation of ICH, the mice were subjected to three

trials at the speed of 40 rpm, with a 5-min rest between each trial. The latency to fall of each trial was recorded. The mice staying on the rod for more than 300 s were removed, and the latency to fall was recorded as 300 s.

Corner Turn Test

The mice of all groups ($n = 9$ per group) were put in a 30° corner, and they will quit through turning to the left side or to the right side. Only those turnings with full rearing along either wall were recorded. After the operation of ICH, the mice tend to turn to the ipsilateral to the damages (21). The test had been performed for 10 times, at an interval of 1 min, and the right turns' percentage was recorded.

Water Content of the Brain

It was determined 3 days after the surgery. Brains ($n = 5$ per group) were collected and dissected into the ipsilateral and contralateral cerebral hemispheres on ice. The wet weight of each cerebral hemisphere was measured using an electric analytic balance. The brain tissues had been dried at 120°C for 24 h until there was no decrease of the weight, and the dry weight was measured. The water content of the brain = (wet weight – dry weight)/wet weight × 100%.

Nissl Staining and Immunohistochemical Staining

The mice were transcardially perfused with normal saline and paraformaldehyde (4%) 3 days after the operation, and their brains were isolated and dehydrated in 15, 20, and 30% sucrose. Then, the brains were cut with a freezing microtome to collect coronal sections with the thickness of 25 μm. Nissl staining was conducted using a cresyl violet (C9140-1; Solarbio) staining kit following the manufacturer's instructions (Solarbio, China). Immunohistochemical staining for glia fibrillary acidic protein (GFAP) ($n = 5$ per group) was conducted for detecting neuroinflammation. Brain sections were soaked in 0.25% Triton X-100 and 3% H_2O_2, blocked in normal goat serum (5%), and then, they had been incubated at 4°C overnight with the primary antibodies anti-GFAP (1:800; Abcam), anti–IL-1β (1:200; Abcam) and FOXO3a (1:200; Abcam), followed by anti–rabbit immunoglobulin G (H + L) (1:200, AS003; Abcam) of horseradish peroxidase goat. Sections were developed with a DAB kit (Zymed Laboratories Inc., San Francisco, CA, USA). The digitized images were obtained with a microscope (Axioplan 2, Zeiss, Oberkochen). The GFAP and IL-1β-positive neurons were counted by means of the optical fractionator and a computer-assisted stereological Olympus Toolbox system. The brain section was first delineated using a 4× objective. A square grid of 150 × 150 μm was randomly superimposed with and a 100 × 100-μm square dissector counting chamber placed on the counting area of the image and moved through all of the counting areas until the whole section was finished.

For the double-immunofluorescence staining, sections were rinsing with phosphate-buffered saline (PBS) for three times and were blocked with normal goat serum for 1 h at room temperature and then incubated with rabbit anti-NeuN (1:400, Abcam) and mouse anti-Iba1 (1:200, Abcam) overnight at 4°C. Then slices were incubated with Alexa Fluor 488–conjugated secondary antibodies (goat anti–mouse, 1:400, GB25301; goat anti–rabbit, 1:400, GB25303; Servicebio) for 1 h at room temperature. The brain slices were stained with DAPI for 5 min. The expression of NeuN and Iba1 in neuron was captured under a fluorescence microscope using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA). The Iba1-positive cells were counted and presented as cells/mm^2.

TUNEL Assay

TUNEL staining was performed to detect neuronal apoptosis in mice of different groups on day 3 after ICH ($n = 5$). Brain sections were washed with PBS and incubated with 20 μg/mL protease K solution. Then brain sections were washed and incubated with the TUNEL reaction mix. TUNEL-positive cells were imaged and calculated under a fluorescence microscope. Data are presented as TUNEL-positive cells/mm^2.

Extraction of RNA and Reverse Transcription–Quantitative Polymerase Chain Reaction

The brain tissue and blood were lysed, and RNA was extracted with Trizol reagent (Invitrogen, USA) and was purified with a purification kit (Thermo Fisher scientific). For detection of the levels of mRNA, 1 μg RNA was reverse transcribed into cDNA using the PrimeScript™ II First-Strand cDNA synthesis kit (Takara Bio, Inc., Dalian, Japan) while using the Mir-XTM miRNA First-Strand Synthesis Kit (Takara Bio, Inc., Dalian, Japan) for miR-144 and miR-451, according to the manufacturer's instructions. The cDNA was amplified with SuperScript III Reverse Transcriptase (Invitrogen, USA). Transcripts were detected with PrimeScript™ RT Master Mix (Takara Bio, Inc., Dalian, Japan) in accordance with the instructions of the manufacturer. The genes' mRNA levels were standardized to the housekeeping gene GAPDH; U6 was used as internal control of miR-144 and miR-451. All genes' primer sequences are presented in **Table 1**. Relative levels of miRNA and mRNA were calculated using $2^{-\Delta\Delta CT}$ method. All the reactions were run in triplicate.

TABLE 1 | The sequences of the primers.

Name of primers	Sequence of primers
Forward-Sod1	5′–TGAAGAGAGGCATGTTGGAG–3′
Reverse-Sod1	5′–CCACCTTTGCCCAAGTCATC–3′
Forward-Sod2	5′–TCATGCAGCTGCACCACAGC–3′
Reverse-Sod2	5′–CCATTGAACTTCAGTGCAGG–3′
Forward-Cat	5′–TCACTGACGAGATGGCACAC–3′
Reverse-Cat	5′–CTGACTCTCCAGCGACTGTG–3′
Forward-Gpx1	5′–CTCAAGTACGTCCGACCTGG–3′
Reverce-Gpx1	5′–TGTCGATGGTACGAAAGCGG–3′
Forward-GAPDH	5′–AAGGTGAAGGTC GGAGTCAAC–3′
Reverse-GAPDH	5′–GGGGTCATTGATGGCAACAATA–3′
MiR-144	5′–UACAGUAUAGAUGAUGUACU–3′
MiR-451	5′–AAACCGTTACCATTACTGAGTT–3′
U6	5′–CGCTTCGGCAGCACATATAC–3′

Western Blot

Perihematomal striatum was rapidly dissected and homogenized in ice-cold lysis buffer 3 days after the operation of ICH. The brain extracts were loaded on 10 or 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Millipore, Massachusetts, USA), which had been blocked with non-fat milk (5%) for 1 h and then had been incubated with primary antibodies against 14-3-3ζ, 14-3-3-β, 14-3-3-θ, 14-3-3-pan (Chemicon International), FoxO3 (Millipore), and β-actin (Abcam Plc, Cambridge, UK) at 4°C overnight. Then, the samples had been incubated with the secondary antibody for 2 h at room temperature, and then the protein bands were visualized with super-enhanced chemiluminescence, later the analysis of the bands' density was performed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Enzyme-Linked Immunosorbent Assay

Perihematomal striatum had been put in the lysis buffer and centrifuged at 4°C for 30 min to collect the supernatant 3 days after the operation of ICH operation. The levels of TNF-α and IL-1β were determined using the commercial enzyme-linked immunosorbent assay kits (PEPROTECH, USA). According to the manufacturer's instruction, homogenates of individual mice were placed into the 96-well plates, and then, the corresponding primary antibodies were put and then had been incubated overnight at 4°C. The primary antibody was removed, and the sample was blocked with 1% bovine serum albumin. The secondary antibody was put and had been incubated for 1 h. The measurement of the optical density was performed with a microplate reader at 450 nm.

SOD and GSH-Px Activity and MDA Content Estimation

The perihematomal tissues were dissected quickly, homogenized, and diluted using precooled 0.01 M PBS ($n = 4$ per group). MDA concentrations were spectrophotometrically detected at 532 nm according to the instructions of the Nanjing Jiancheng Kit. Brain tissue homogenate was centrifuged at 1,500*g*, 10 min, at 4°C, to collect the supernatant for analyzing the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). GSH-Px activity was determined by absorbance at 412 nm, while SOD activity at 560 nm according the protocols of the commercial test kits (Jiancheng, Nanjing).

Collection of Blood Samples

The peripheral blood samples were obtained from the patients with acute cerebral hemorrhage and the healthy controls who were registered in Northern Jiangsu People's Hospital, which Medical Ethics Committee approved the collection of all blood samples. The serum samples of the patients ($n = 20$) were collected 12 h after admission, and the serum samples of the healthy controls ($n = 18$) were collected 1 day after admission. The serum was collected through centrifuging at 1,500 g/min for 10 min.

Statistical Analysis

All data were analyzed using version 19.0 of SPSS Software (SPSS Inc., Chicago, IL, USA). The data are shown as the mean ± standard error of the mean. The comparisons between each group were analyzed by two-way analysis of variance through *post hoc* Newman–Keuls test. $P < 0.05$ was considered as statistically significant.

RESULTS

Time Course of the Expression of miR-144/451 in Perihematomal Area Following ICH

To detect the levels of miR-144/451 after ICH, we checked their expressions in the perihematomal area obtained from the mice with ICH. The mRNA levels of miR-144/ 451 were analyzed through reverse transcription–quantitative polymerase chain

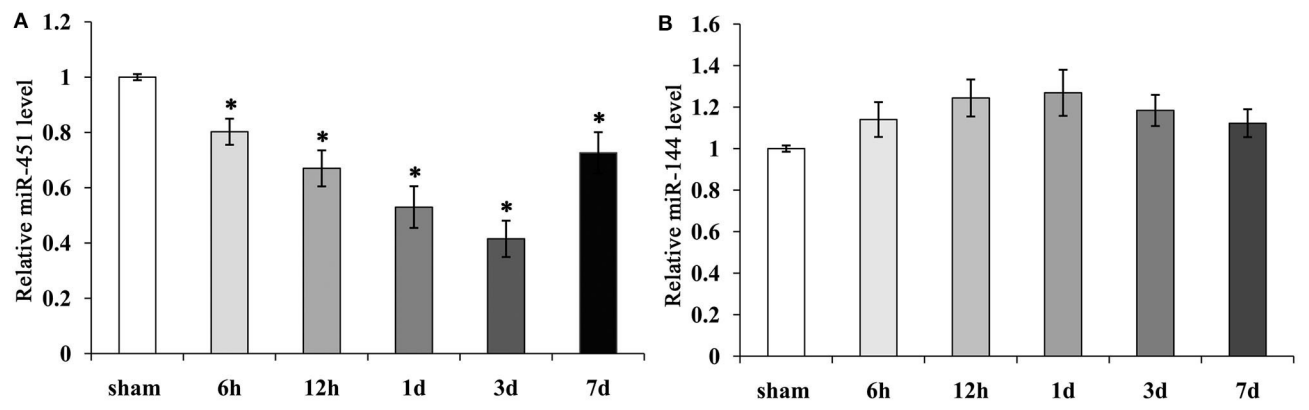

FIGURE 1 | Expression of miR-144 and miR-451 in the peri-hematomal area following ICH. **(A)** miR-451 gradually declined significantly from 6 h to 3 days, then recover at 7 days following ICH, Sham group was used as a control. **(B)** miR-144 increased slightly from 6 h to 1 day, then decreased to that of sham-operation group. Five mice were included in each group. Data were presented as the mean ± standard deviation ($n = 9$). *$p < 0.05$ vs. the sham group. miR, microRNA; ICH, intracerebral hemorrhage.

reaction (qRT-PCR) analysis, and it was found that compared with the sham group, miR-451 gradually declined from 6 h to 3 days and then recovered at 7 days following ICH ($P < 0.05$, **Figure 1A**). The level of miR-144 increased slightly from 6 h to 1 day, followed by a decrease to that of the sham-operation group with $P > 0.05$, shown in **Figure 1B**.

KO of miR-144/451 Deteriorated the Neurological Deficits and Brain Edema of ICH Mice

Next, we applied the model of miR-144/451 KO to establish the mice model with ICH to learn more about the importance of the cluster of miR-144/451 after ICH. The corner test, rotarod test, and cylinder test were used for testing neurological deficits in this study. The mice with ICH exhibited significant neurological damages 24 h after ICH, indicating that the model with ICH was established successfully. According to **Figure 2A**, Nissl staining showed a hematoma area at 3 days after the operation of ICH, and the hematoma area expanded in the ICH-KO group through comparing with the mice with ICH ($P < 0.05$, **Figure 2A**). The TUNEL assay was carried out to determine the neuronal apoptosis in the perihematomal area of different groups (**Figure 2B**). The number of TUNEL-positive cells increased significantly in the perihematomal area of the ICH mice (**Figure 2B**). KO of miR-144/451 cluster significantly aggravated the neuronal apoptosis in ICH mice ($P < 0.01$, **Figure 3B**).

Compared with the ICH group, the time spent on the rod and frequency of the placements of the left paw were significantly reduced while the right turns of the mice with ICH increased through comparing with the sham group from day 1 after ICH, with $P < 0.05$ shown in **Figures 2C–E**, and all the neurological parameters were exacerbated in the KO + ICH group obviously with $P < 0.05$ shown in **Figures 2C–E**, suggesting that the KO of miR-144/451 worsened neurological damages in ICH.

As brain edema is an important biological event in brain injuries after ICH, we determined the water content of the brain of the mice using the wet/dry method. The water content of the brain of the mice was significantly increased after the operation of ICH through comparing with the sham group, which was exacerbated in the mice with miR-144/451KO ICH ($P < 0.05$, **Figure 2F**).

The Pearson correlation test was applied to analyze the association of the expression levels of miR-144/451 and the corner test and cylinder test. miR-451 revealed a positive correlation with frequency of the placements of the left paw (**Figure 2G**, $r^2 = 0.37$, $P < 0.01$) and a negative correlation with right turns (**Figure 2G**, $r = 0.52$, $P < 0.01$) without any significant correlation between the level of miR-144 and the behavior test (**Figure 2G**, $p > 0.05$). This indicated that miR-451, not the miR-144, might play the main regulatory role in the brain injuries after ICH.

KO of miR-144/451 Exacerbated Hemin-Induced Inflammatory Response

An increasing evidence indicates that inflammatory response is crucial during the development of ICH. ICH activated the GFAP-positive astrocytes and protein level around the hematoma, and miR-144/451 KO markedly exacerbated the number of $GFAP^+$ neurons ($P < 0.05$, **Figures 4A,B**). Activation of microglia also contributes to neuronal injury after ICH. We performed the double-immunofluorescence labeling Iba1 (red) and NeuN (green) to detect the activation of microglia in the perihematomal area of mice. Results showed that the activated microglia is markedly increased in mice after ICH operation; miR-144/451 KO obviously increased the number of Iba1-positive neurons in ICH mice ($P < 0.05$, **Figure 4C**). We also investigated the secretion of proinflammatory cytokines and found that the depletion of miR-144/451 markedly upregulated the levels of TNF-α and IL-1β in the brains of the mice with ICH ($p < 0.05$, **Figures 4D,E**). Spearman correlation analysis was applied to detect the correlation between the expression of miR-144/451 and the levels of TNF-α and IL-1β. It presented a negative correlation between the expression of miR-451 and the levels of TNF-α and IL-1β with $P < 0.01$ shown in **Figure 4F** without a significant correlation between the expression of miR-144 and levels of the TNF-α and IL-6 ($p > 0.05$, **Figure 4F**).

KO of miR-144/451 Promoted Oxidative Stress in ICH Mice Brain

It is well-known that excessive oxidative stress promotes neuronal injuries in models with ICH (20). This study detected whether the cluster of miR-144/451 could modulate the hemin-induced oxidative stress. We detected the levels of biomarkers of oxidative stress, namely, SOD and MDA, as well as GSH-Px in the perihematomal area. The results showed that the levels of MDA increased significantly, while the activities of SOD and GSH-Px decreased significantly in the mice with ICH through comparing with the sham group, and the depletion of the cluster of miR-144/451 in the mice with ICH promoted these changes significantly (**Figures 5A–C**, $p < 0.05$). As a previous study reported, miR-451 could suppress oxidative stress by targeting 14-3-3ζ, which promoted the expression of two antioxidant genes-cat and gpx. We also detected some oxidative stress genes, including cat, gpx1, sod1, and sod2. The results revealed that the FoxO3-regulated antioxidant gene cat, gpx1, and sod1 were down-regulated significantly in the mice with ICH through comparing with the sham group, $p < 0.05$, shown in **Figure 3C**, and the depletion of miR-144/451 markedly worsened the decreasing of Gpx1 and cat, $p < 0.05$, shown in **Figure 3C**. The levels of SOD2 under the operation of ICH and the depletion of miR-144/451 had no significant change.

miR-144/451 Repressed the miR-451/14-3-3ζ Axis Pathway in Mice With ICH

Our previous studies have revealed that miR-451 suppresses oxidative stress through targeting the axis of the miR-451 and 14-3-3ζ as well as FoxO3 (13). To determine whether the axis of the miR-451 and 14-3-3ζ as well as Foxo3 is involved in ICH, this study determined the protein levels of 14-3-3ζ and Foxo3. As presented in **Figures 3A,B**, after the operation of ICH, the protein levels of 14-3-3ζ down-regulated, while Foxo3

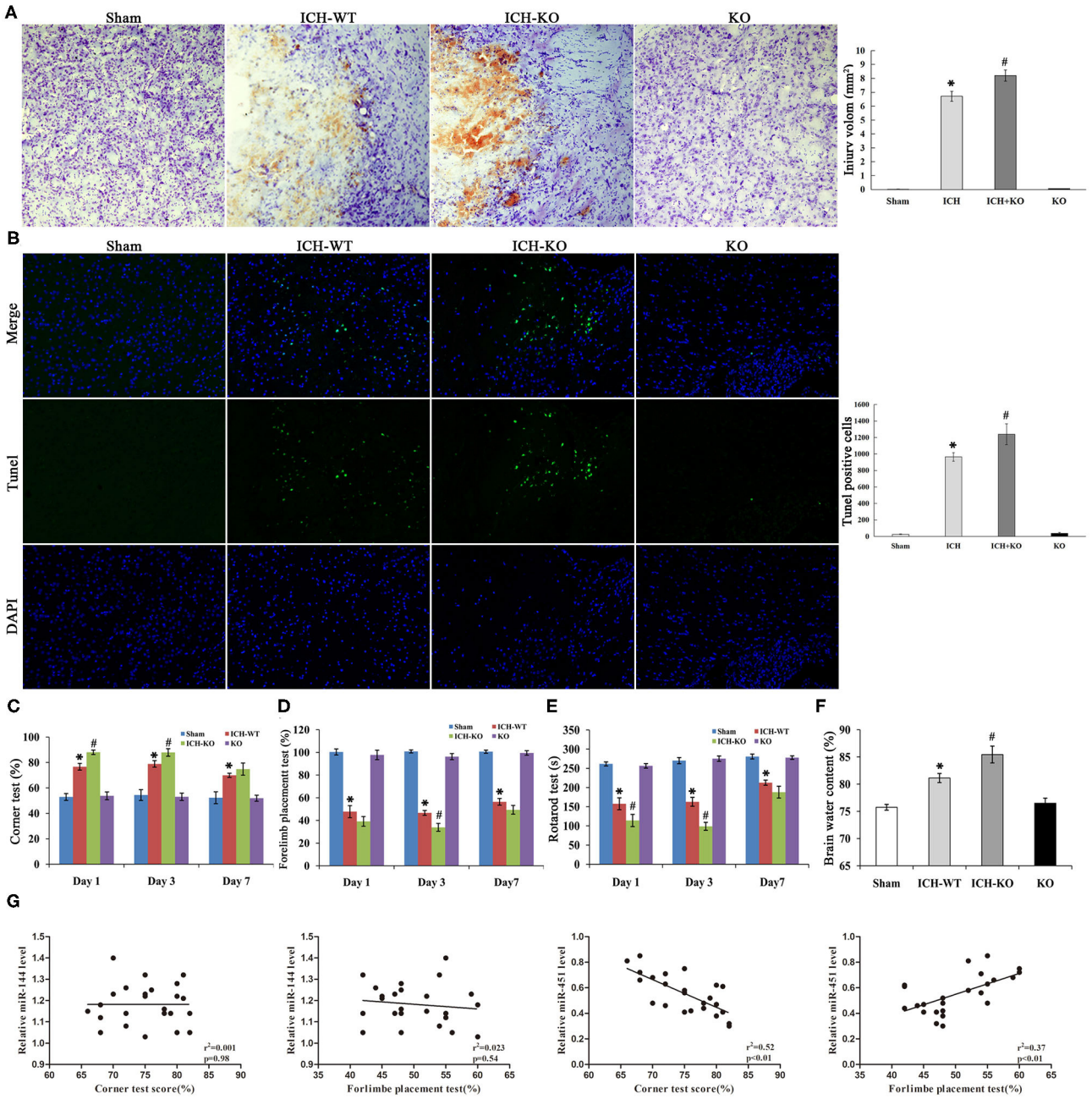

FIGURE 2 | Knockout of miR-144/451 cluster aggravated neurological function and brain injury of ICH mice. **(A)** Representative Nissl stained images of hematoma area on day 3 after ICH ($n = 5$). **(B)** Representative images of TUNEL (green) positive neurons in peri-hematomal area of mice in different groups ($n = 5$). **(C)** Corner tests were performed on days 1, 3 and 7 ($n = 9$). **(D)** Forelimb placement tests were performed on days 1, 3 and 7 ($n = 9$). **(E)** The time on the rod in the rotarod test on days 1, 3 and 7 ($n = 9$). **(F)** Brain water content was measured on day 3 after ICH ($n = 5$). **(G)** Correlations between miR-144, miR-451 levels and behavior tests using the Pearson correlation test. Data were presented as the mean ± standard deviation. *$p < 0.05$ vs. the sham group; #$p < 0.05$ vs. the ICH group. miR, microRNA; ICH, intracerebral hemorrhage; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

up-regulated in the brains of the mice with ICH through comparing with the sham group. The depletion of miR-144/451 significantly up-regulated the expression of 14-3-3ζ and Foxo3 compared with the mice with ICH (**Figures 3A,B**; $p < 0.05$). KO of the cluster of miR-144/451 did not affect the other subtypes of 14-3-3 protein in mice with ICH. We also detected the expression and location of FoxO3 in the area around the hematoma, as presented in **Figure 3D**, the FoxO3 transported from cytoplasm to nucleus after ICH operation; KO of miR-144/451 promoted this procession obviously.

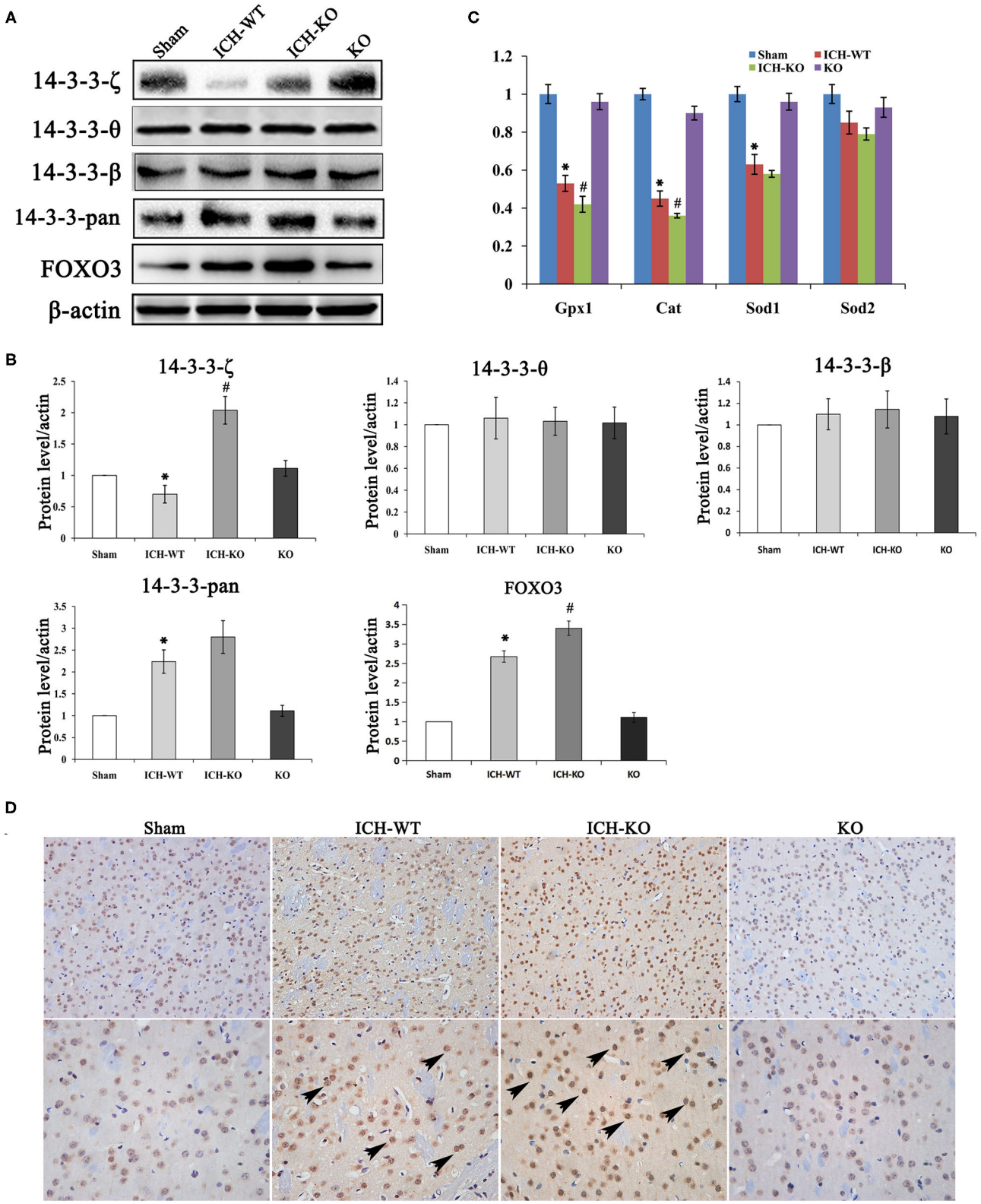

FIGURE 3 | miR-144/451 cluster regulated the expression of 14-3-3ζ and FoxO3 in ICH mice. **(A,B)** The protein expression of 14-3-3ζ, 14-3-3-β, 14-3-3-θ, 14-3-3-pan, Fox3 in the peri-hematomal area of mice in different groups. **(C)** The mRNA levels of genes in the downstream of 14-3-3ζ, including cat, gpx1, sod1, and sod2 in different groups. **(D)** Representative microphotographs showing FoxO3-positive cells in peri-hematomal area on day 3 after ICH. *$p < 0.05$ vs. the sham group; #$p < 0.05$ vs. the ICH group. miR, microRNA; ICH, intracerebral hemorrhage; FoxO3, forkhead box.

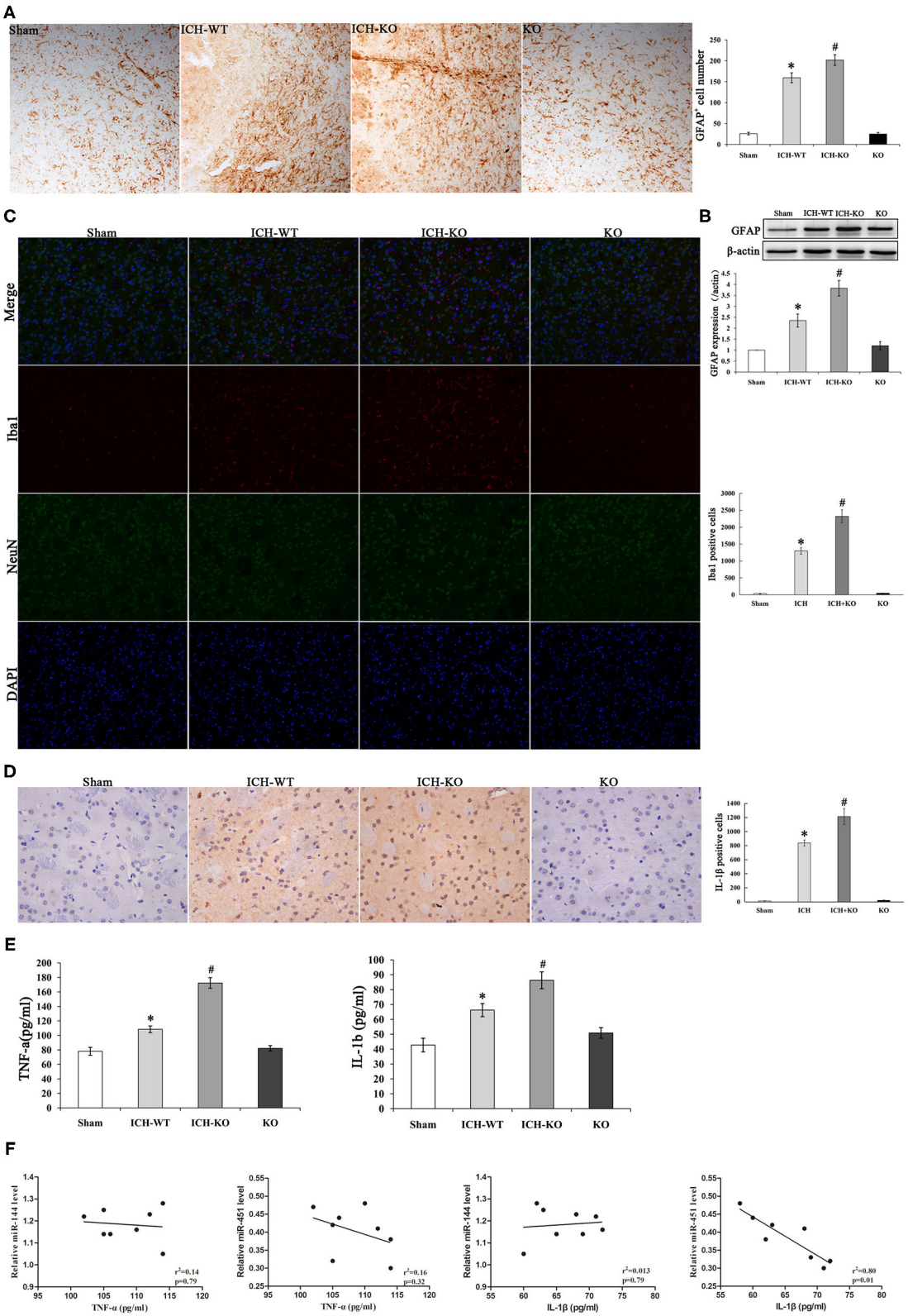

FIGURE 4 | Knockout of miR-144/451 cluster exacerbated neuroinflammation and promoted pro-inflammatory cytokines after ICH. **(A)** Immunohistochemistry for GFAP in peri-hematomal area of mice in different groups ($n = 5$). **(B)** Western Blotting for GFAP in brain of different groups. **(C)** Representative images of double immunofuorescence labeling Iba1 (red) and neuronal nuclei (NeuN)-positive neurons (green) in peri-hematomal area of mice in different groups. **(D)** Representative

(Continued)

FIGURE 4 | microphotographs showing IL-1β-positive cells in peri-hematomal area on day 3 after ICH. **(E)** Elisa assays for TNF-α and IL-1β in perihematomal area of different groups ($n = 5$). **(F)** Correlations between miR-144, miR-451 levels and pro-inflammatory cytokines using the Spearman's correlation test. Data were presented as the mean ± standard deviation. *$p < 0.05$ vs. the sham group; #$p < 0.05$ vs. the ICH group. miR, microRNA; ICH, intracerebral hemorrhage; GFAP, glial fibrillary acidic protein; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; Iba1, Ionized calcium binding adaptor molecule 1; NeuN, neuronal nuclei.

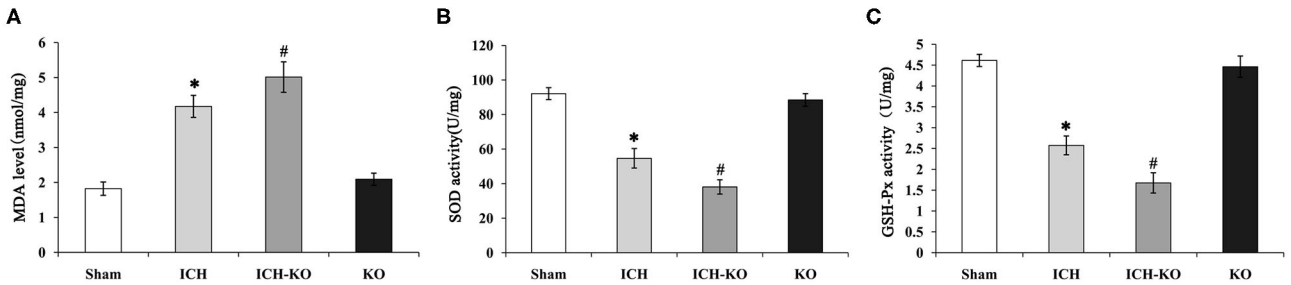

FIGURE 5 | Knockout of miR-144/451 cluster promoted oxidative stress in ICH mice. 3 days after ICH induction, the levels of oxidative stress biomarkers, including **(A)** MDA, **(B)** SOD, and **(C)** GSH-Px were measured in the peri-hematomal area of mice in different groups. Data were presented as the mean ± standard deviation ($n = 4$–5). *$P < 0.05$ vs. the Sham group; #$P < 0.05$ vs. the ICH group. miR, microRNA; ICH, intracerebral hemorrhage; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase. *$p < 0.05$ vs. the sham group; #$p < 0.05$ vs. the ICH group.

TABLE 2 | Clinical characteristics of the ICH patients and control group who were used for analyzing the expression of the miR-144 and miR-451 (19).

	ICH patients	Healthy control	*P*-value
Ethnicity, (%)	100%	100%	1
Age, (years, mean + SD)	59.8 ± 7.4	62.5 ± 4.2	0.89
Sex, male, (%)	63%	56%	0.53
Hypertension, (%)	56%	40%	0.72
Diabetes, *n*	2	3	0.42
Body mass index, (kg/m^2, mean + SD)	26.6 ± 8.4	23.5 ± 4.1	0.36
Hours since ICH, (h, mean + SD)	40.6 ± 7.4	N/A	<0.001

Circulating Levels of miR-144/451 of the Patients With ICH

We detected the miR-144/451's mRNA levels of the patients with ICH and the healthy controls using the qRT-PCR assay. The clinical characteristics of the ICH patients and control group were described in **Table 2** (19). As shown in **Figure 6A**, circulating miR-451 decreased significantly ($p < 0.05$), while the miR-144 increased to a lesser extent ($p > 0.05$) in the patients with ICH compared with the controlled patients.

DISCUSSION

ICH, accounting for 10–30% of all stroke types, is one of the major health burdens worldwide (22). ICH survivors frequently suffer from left hemiplegia, speech disorders, and vascular cognitive impairments. To date, there is no effective clinical treatment to alleviate ICH and post-ICH complications (2). Accumulating evidences showed that various miRNAs were identified as biomarkers for diagnosing and the progression of neurological diseases (miR-21-P) (8–11).

miR-144/451 has been reported to be involved in the development of various diseases. Previously, it was found that the miR-451's knockdown in the zebra fish's embryos impaired erythroid maturation significantly (13). Several groups reported that miR-144/451 was critical for suppressing cell survival in several kinds of cancers, including lung cancer, gastric cancer, and so on (14–18). For the neurological diseases, miR-144 elevated in the early stage of AD patients and induced the depletion of ADAM10, which forced metabolism of amyloid β-peptide (Aβ) to protect the brain. Overproduced Aβ recruited the transcription factor AP-1 and GATA and consequently promoted the expression of miR-144, forming the vicious circle (23). Therefore, we hypothesized that miR-144/451 might be involved in the neuronal injuries post the ICH conditions. In the present study, collagenase IV significantly suppressed the expression of miR-451 while just increasing the level of miR-144 slightly in the perihematomal area. Besides, the KO of miR-144/451 aggravated the motor deficits, neuronal apoptosis, and the brain edema significantly in the mice with ICH, proven by worsened behavioral readouts, increased TUNEL-positive cells, and increased water content of the brain. But on day 7, the role of miR-144/451 is not obvious; this may be because miRNAs play its role when subjected to intense stimulation (13). Fu et al. proved that miR-451 mimics improved the neurological deficits induced by cerebral ischemia/reperfusion in stroke mice (19). Despite more emerging evidences showed the protective roles of miR-144/451, several other studies showed that they exaggerated brain injuries in special neurological diseases (23–26). For example, it showed that the KO of miR-451 alleviated the behavior deficits and apoptosis of hippocampus in mice with KA-induced epilepsy by upregulating GDNF level (26). This contradiction might be related with miR-144/451 targeting different genes in different pathologic conditions. Moreover, only Nissl staining is applied to detect the perihematomal edema in ICH mice in this study.

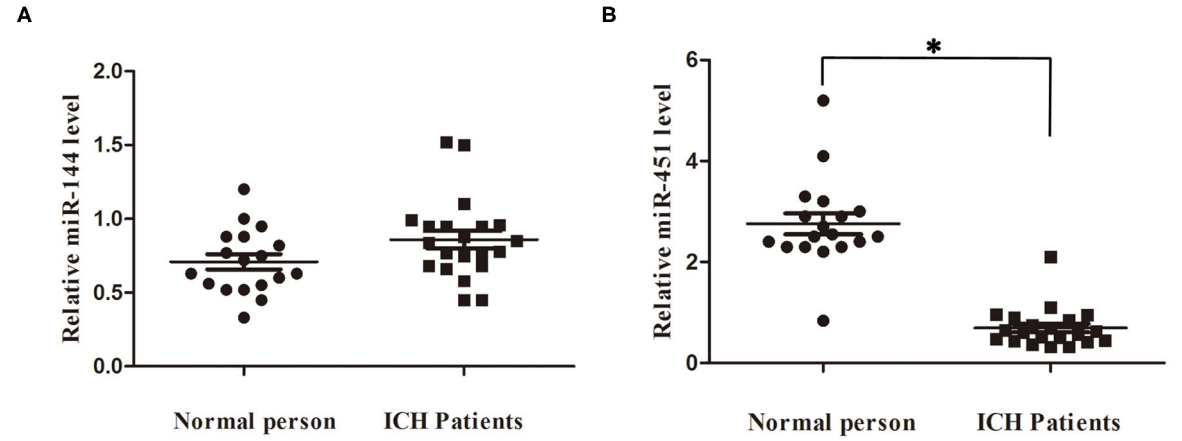

FIGURE 6 | Serum miR-144 and miR-451 was elevated in ICH patients. **(A)** miR-451 declined significantly at 7 days after ICH compared with control people. **(B)** miR-144 showed no significantly change at 7 days after ICH compared with control people. *$p < 0.05$ vs. the sham group. miR, microRNA; ICH, intracerebral hemorrhage.

T2-weighted magnetic resonance (MRI) is a better way to detect brain edema in ICH mice *in vivo*. However, the tissue loss volume would be more ambiguous compared with histology assessment (5 μm), because of the slice thickness of MRI (2.0 mm). The images from MRI cannot show clear anatomical definition of the brain area including striatum, hippocampus, and white matter structures. The future study will apply both the macroscopic timed imaging modalities and histology to measure the brain edema in different time points in ICH mice. We further detected the miR-144/451's levels in clinical trials; miR-451 decreased in sera from the patients with ICH, whereas miR-144 showed no significant change in the patients with ICH compared with the normal persons. Our preclinical and clinical data suggested that higher level of the cluster of miR-144/451 indicated a good prognosis of prediction in the patients with ICH.

Oxidative stress under ICH conditions is detrimental to neurons and deeply involved brain injuries in models or patients with ICH (1). A better understanding of the mechanisms of ICH-induced oxidative stress could offer promising strategy for treating ICH (2). As our laboratory has reported, miR-451 protects against oxidative stress in erythroid cells by targeting 14-3-3ζ which sequesters the transcription factor Foxo3 to cytoplasm and allows the transcription of two antioxidant genes, namely, cat and gpx1 (13, 27). The present observations showed that the levels of ROS and GPX produced in the mice with miR-144/451-KO ICH decreased, while MDA increased compared with that of the mice with ICH-wide type (WT), this may indicate a mechanistic interpretation for the protective effects of miR-144/451 in ICH. Furthermore, the KO of miR-144/451 in the mice with ICH reduced the antioxidant ability of neurons by activating 14-3-3ζ, thus inhibiting the transcription of cat and gpx1. The results in this study clearly show that miR-144/451 existing around the perihematomal area protects the survival neurons against oxidative stress. Consistently, Wang et al. found that the depletion of miR-144/451 promoted the ischemia/reperfusion–induced oxidative stress by activating the pathway of CUGBP2-COX-2 (28). It suggested that the cluster of miR-144/451 played a profound inhibitory effect on oxidant injuries and facilitated neuronal survive in brains of mice with ICH. However, another study demonstrated that antagomiR-451 could inhibit oxidative stress induced by OGD/R through the activation of AMPK signaling in neurons (29).

Neuroinflammation in ICH is evoked by hematoma degradation products, including the hemin, fibrin, and thrombin. The blood components promote microglia activation and the secretion of proinflammatory cytokines (1, 3). Previous studies have demonstrated that miR-451 inhibited the neuroinflammation in chronic inflammatory pain through suppressing microglia activation via targeting TLR4 (30). In our study, the KO of miR-144/451 in the mice with ICH markedly upregulated the secretions of IL-1β and TNF-α around hematomas, together with the number of GFAP and Iba1-positive neurons. These results are in line with the anti-inflammatory effects of miR-144/451 observed in the other diseases (17, 21). Chung et al. reported that the KO of miR-451 deteriorated the peribronchial inflammation in the airways of allergen-challenged mice (21). We further detected the relationship between the level of miR-451 and the levels of proinflammatory cytokines, such as IL-1β and TNF-α, and found a negative correlation between them. These results suggested that functional impairment of miR-144/451 could exaggerate human brain injuries by promoting the neuroinflammation in the patients with ICH.

FoxO3, highly expressed in brain tissue, plays crucial roles in cell proliferation, differentiation, and oxidative stress as transcription factor (30). When subjected to stimulation, FoxO3 is activated and binds to the 14-3-3 proteins in the nucleus, inhibiting the FOXO3 transcription (31). Li et al. revealed that FoxO3 has the potential to promote neuronal damage following cerebral ischemia, indicating suppression of FoxO3a may protect neurons against ischemic injury (32). Our findings illustrated that the expression of 14-3-3ζ is suppressed, whereas the level of FoxO3 increased in response to ICH injury; loss of miR-144/451 upregulated levels of both proteins. Immunostaining confirmed

that ICH operation leads to FoxO3 translocating from the cytosol to nucleus; KO of miR-144/451 promoted this procession. Yu et al. reported that loss of miR-451 promoted accumulation of 14-3-3z and inhibited the activity of FoxO3 and its downstream genes including SOD and CAT (13). This different behavior of miR-144/451 might be related with the different mechanism in brain and erythrocytes.

In conclusion, we applied an easily reproduced mice model with miR-144/451 KO to show that loss of the cluster of miR-144/451 worsened the neurological function deficits and brain injuries in mice with ICH. Loss of the cluster of miR-144/451 promoted the overgeneration of ROS via activating the regulatory axis of miR-451-14-3-3ζ, which resulted in the death of neuron in the pathogenesis of ICH. Additionally, the cluster of miR-144/451 also highlighted the modulation on neuroinflammation and oxidative stress following ICH. Interestingly, we identified that miR-451 was dominant for regulating the ICH. Future studies will establish a single model with miR (-144 or−451) KO to test the exact importance of miR-144/451 in brain injuries during ICH.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The human studies were reviewed and approved by the Medical Ethics Committee of the Subei Hospital, Yangzhou, China. Written informed consent was provided by the patients for participation in the study, or where necessary, their next of kin. The animal studies were reviewed and approved by the Institutional Animal Care and Use Committee and the Animal Ethics Committee of Yangzhou University [SYXK (Su) IACUC 2017-0045].

AUTHOR CONTRIBUTIONS

JX, XW, and YHo conceived and designed the study. JX and DY obtained the fundings. LW and XD performed animal experiments. YHu, YS, YW, and ZD collected patients' blood samples and contributed data and analysis. CW and DY contributed generation of the manuscript. All authors contributed to the editing of the manuscript.

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