Oxymatrine ameliorates white matter injury by modulating gut microbiota after intracerebral hemorrhage in mice

Jing Li
Clinical Biobank Center, Microbiome Medicine Center, Department of Laboratory Medicine, Zhujiang Hospital, Southern Medical University

Jianhao Liang
Clinical Biobank Center, Microbiome Medicine Center, Department of Laboratory Medicine, Zhujiang Hospital, Southern Medical University

Meiqin Zeng
Clinical Biobank Center, Microbiome Medicine Center, Department of Laboratory Medicine, Zhujiang Hospital, Southern Medical University

Kaijian Sun
Clinical Biobank Center, Microbiome Medicine Center, Department of Laboratory Medicine, Zhujiang Hospital, Southern Medical University

Yunhao Luo
Clinical Biobank Center, Microbiome Medicine Center, Department of Laboratory Medicine, Zhujiang Hospital, Southern Medical University

Huaping Zheng
The Neurosurgery Institute of Guangdong Province, Zhujiang Hospital, Southern Medical University

Feng Li
The Neurosurgery Institute of Guangdong Province, Zhujiang Hospital, Southern Medical University

Wen Yuan
Laboratory Animal Center, Zhujiang Hospital, Southern Medical University

Hongwei Zhou
Clinical Biobank Center, Microbiome Medicine Center, Department of Laboratory Medicine, Zhujiang Hospital, Southern Medical University

Junshan Liu
Department of Pharmacology of Chinese Medicine, School of Traditional Chinese Medicine, Southern Medical University

Haitao Sun (✉ 2009sht@smu.edu.cn)
Clinical Biobank Center, Microbiome Medicine Center, Department of Laboratory Medicine, Zhujiang Hospital, Southern Medical University
Abstract

Background

White matter injury (WMI) significantly affects neurobehavioral recovery in intracerebral hemorrhage (ICH) patients. Gut dysbiosis plays an important role in the pathogenesis of neurological disorders. Oxymatrine (OMT) has therapeutic effects on inflammation-mediated diseases. Whether OMT exerts therapeutic effects on WMI after ICH and the role of gut microbiota in this process is largely unknown.

Methods

ICH model was established by collagenase IV injection. OMT was daily administrated via oral gavage after ICH. Neurological deficits, WMI, the severity of corticospinal tract (CST) injury, intestinal barrier function and systemic inflammation were investigate after ICH. Microbial 16S rRNA sequencing was performed to reveal the dynamic microbial profiles. Fecal microbiota transplantation (FMT) was performed to elucidate the role of gut microbiota in the pathogenesis of ICH.

Results

OMT promoted a better long-term neurological function recovery and ameliorated axonal demyelination, microgliosis and glial scar formation in the peri-hematoma region and distal CST in the chronic phase after ICH. The significant and persistent alterations of gut microbial composition induced by ICH, which lasted more than two weeks, were obviously regulated by OMT via increasing the species richness and diversity. Additionally, treatment with OMT alleviated intestinal barrier dysfunction, accompanied by a significant down-regulation of the levels of pro-inflammatory cytokines. Correlation analysis revealed that gut microbiota alteration was correlated with inflammation, intestinal barrier permeability, and neurological deficits after ICH. Moreover, the therapeutic effects of OMT on ICH-induced WMI and intestinal barrier disruption were transferrable by fecal microbiota transplantation (FMT).

Conclusion

Our study showed that OMT ameliorates ICH-induced WMI, neurological deficits and intestinal barrier disruption, and OMT-modulated gut microbiota plays an important role in the underlying mechanism.

Background

Intracerebral hemorrhage (ICH) is the second most common subtype of stroke (10–15%) with high morbidity and mortality, remaining a major cause of death and disability globally[1]. A series of pathological changes, such as the mass effect of hematoma and hemorrhage-induced secondary injury, including neuronal death, demyelination, axonal degeneration, and glial scarring in the peri-hematoma
area, occur after ICH[2]. White matter consists of axons, myelin sheaths, and supporting glial cells. Due to its crucial role in neural signal transmission, both subcortical white matter injury (WMI) and distal white matter fiber tracts disruption produce severe neurological deficits[3, 4]. A clinical study reported that WMI was a common pathological event in patients with lobar ICH (present in 77% of patients)[5], which is highly related to the motor impairment[6]. Interestingly, WMI displays potential repairable property, which raises emerging attention from more researchers. However, more attention was paid to the progression and repairment of axonal degeneration and demyelination around primary brain lesions, and little was known about the pathological changes of whiter matter fiber tracts of the spinal cord, especially the corticospinal tract (CST), as well as its role in neurobehavioral recovery. The severity of ipsilateral CST injury was closely associated with the activity of daily living impairment in ICH patients[7]. Our previous work had firstly revealed that striatum hemorrhage induced progressive destruction of the ultrastructure integrity of the CST at the cervical enlargement under microscope, which could prolong for at least 5 weeks[8].

Accumulating evidence implicates intestinal bacteria is a crucial factor for the bidirectional communication between the gut and central nervous system [9]. Supplementation with a complex microbiota or their fermentation products significantly rescued the immature phenotype and impaired function of microglia in germ-free mice[10]. Moreover, gut microbiota is an important regulator for modulating microglia-mediated neuroinflammation under disease conditions [11]. Mounting clinical and preclinical evidence elucidates that gut microbiota is a potential environmental factor for stroke outcome. Ischemic stroke patients and transient ischemic attack patients displayed an obvious dysbiotic microbiota, characterized by a lower abundance of beneficial commensal taxa and a higher abundance of opportunistic pathogens[12]. In a traumatic spinal cord injury mouse model, spinal contusion injury induced persistent gut dysbiosis and intestinal barrier dysfunction, resulting in bacteria translocation, immune homeostasis disruption, and behavioral impairment deterioration[13]. However, the study on the signature of gut microbiota after ICH is still insufficient. In an animal model, ICH caused significant gut dysbiosis and gastrointestinal function impairment, and bacteriotherapy by transplanting with healthy microbiota improved neurological function recovery by modulating immune response[14]. In our previous study, selectively inhibiting NLRP3 inflammasome was sufficient to modulate the gut microbial composition, ameliorate CST injury of the cervical enlargement and alleviate neurological deficits after ICH[15]. Therefore, gut microbiota may be an important therapeutic target for the progression of ICH. However, the study on the signature of gut microbiota after ICH is still insufficient.

Oxymatrine (OMT) is a main quinolizidine alkaloid extracted from the traditional Chinese herb Sophorae Flavescentis Radix. Emerging studies demonstrated that OMT conferred neuroprotective effects on several neurological disorders, such as ischemic stroke and spinal cord injury. In preclinical models, OMT protected against cerebral ischemia-reperfusion injury by inhibiting pro-inflammatory signaling pathways[17]. It was reported that OMT could inhibit the progression of acute spinal cord injury by modulating inflammation, oxidative stress, and apoptosis through the TLR4/NF-κB pathway[18]. However, whether OMT exerted therapeutic effects on WMI after ICH and the role of gut microbiota in these processes are largely unknown.
Therefore, the present study aimed to investigate whether OMT could attenuate hemorrhage-induced WMI, especially secondary CST injury in cervical enlargement, and the role of gut microbiota in the underlying mechanism in the ICH model. We hypothesized that OMT alleviated ICH-induced WMI and neurobehavioral deficits by modulating gut microbiota.

Materials And Methods

Animals

Adult (10–12 weeks) male C57BL/6J mice, weight 25–30 g at the time of ICH operation, supplied by the Laboratory Animal Center of Southern Medical University, were housed under standard 25°C housing conditions with regular 12h light/dark cycles. All the animals were free access to food and water. All experimental procedures were approved by the Institutional Ethics Committee of Zhujiang Hospital of Southern Medical University and conducted according to the guidelines of the National Institute of Health Laboratory Animal Care and Use Guidelines.

Model Of Intracerebral Hemorrhage

ICH model was conducted as previously described[8]. Briefly, anesthesia was performed by intraperitoneal injection of tribromoethanol (0.02 ml/g, Rhone, Merieux, Harlow, UK) and then the animal was placed in a stereotactic frame with the head secured. A 1-cm midline incision was made to expose the skull after disinfecting the scalp. Thereafter, a 1-mm-diameter burr hole was drilled to implant a 0.5 ul syringe needle into the right striatum (stereotaxic coordinates: 0.2 mm anterior and 2.0 mm lateral to bregma, 3.5 mm in depth). Type IV bacterial collagenase (Sigma, St. Louis, MO, USA; 0.075 U in 0.4 ul of saline) was slowly injected into the striatum at a rate of 0.05 ul/min over 2 min with an infusion pump to induce intracerebral hemorrhage. Sham controls had only a needle insertion. The needle was left in place for an additional 10 min to avoid reflux before being slowly withdrawn. The incision was sutured after sealing the burr hole with bone wax. Body temperature was maintained through surgery until all mice recovered from anesthesia.

Drug Preparation And Administration

Animals were randomly assigned into three groups, namely Sham, ICH + Vehicle, and ICH + OMT group (Fig. 1A). The mice of the ICH + OMT group daily received OMT (Cat#: A111286, Aladdin) by oral gavage, which was dissolved in deionized water to achieve the concentration of 40 mg/ml, at 120 mg/kg body weight according to previous research [17], until sacrificed. Both the Sham group and the ICH + Vehicle group were given equivalent volumes of deionized water in the same manner.

Brain Water Content Measurement
The brain water content was determined by the wet-dry method[19]. Briefly, at 3 days after ICH, brain samples were harvested and divided into three parts (ipsilateral hemisphere, contralateral hemisphere, and cerebellum) and then weighed to obtain the wet weight. The samples were further placed into an 80 °C oven to dry for 5 days and reweighed to obtain the dry weight. The proportion of brain water content (%) was determined as followed: \[
\frac{(\text{wet weight} - \text{dry weight})}{\text{wet weight}} \times 100%.
\]

**Determination Of The Hematoma Volume**

Hematoma volume measurement was conducted at 3 days after ICH onset. All mice were intracardially perfused with 40 ml ice-cold phosphate-buffered saline (PBS) under deep anesthesia. Afterward, the whole brains were carefully removed and sectioned into 1-mm-thickness coronal slices which were digitalized. The hemorrhage areas on each section were manually drawn and integrated along the anteroposterior axis to calculate the cubic volume of hematoma as followed: hematoma volume (mm$^3$) = area of every section (mm$^2$) × section number × thickness (mm) by the ImageJ software.

**Mnss Test**

The modified Neurological Severity Score (mNSS) test was performed blindly on day 1, day 3, day 7, and day 14 after ICH to examine neurological function[20]. The mNSS score ranges from 0 to 18, with a score of 0 indicating normal and higher scores implying more critical neurological impairment. The neurological function is mainly reflected in sensory (visual, tactile, and proprioceptive), motor (muscle status and abnormal movement), reflex, and balance tests.

**Corner Turn Test**

The corner turn test was conducted by two researchers who were blind to the whole experimental design to evaluate sensorimotor dysfunction after ICH operation[19]. Briefly, the mice were placed into a corner with a 30° angle assembled by two walls made of plastic fiberboards. The animal would generally turn left or right to escape the corner, and the options of direction were recorded after 10 repeated trials with 30 s intervals for each mouse. Only turns involving a full rearing along either wall were recorded. Generally, a mouse could turn either to the left or the right to exit the corner without turning bias, whereas a mouse suffering from ICH preferentially turns towards the unimpaired (i.e., right) body side. The percentage of right turns was calculated as followed: (the times of right turns/total trials’ times) × 100%.

**Measurement Of Intestinal Barrier Permeability**

The permeability of the intestinal barrier was investigated by assessing the circulating level of fluorescein[14]. Briefly, mice were fasted overnight before oral administration with 4 kDa fluorescein isothiocyanate (FITC)-dextran (FD4, 80 mg/ml, Cat#: FD4, Sigma-Aldrich). Blood samples were obtained
4 hours after FD4 gavage and centrifuged for 15 minutes (4000 rpm, 4 °C). The upper supernatant was transferred into a new tube and used for serum FD4 concentration detection. Serum samples were used for FD4 assessment with a plate reader (excitation/emission 480 nm/530 nm). The circulating level of FD4 was detected in duplicate with the plate reader (excitation/emission 480 nm/530 nm) and calculated by establishing a standard curve.

**Immunofluorescence Staining**

At 3 days and 14 days after ICH onset, brain and spinal cord samples were carefully isolated for histological analysis. Briefly, under deep anesthesia, mice were intracardially perfused with PBS followed by 4% paraformaldehyde (PFA). Brain and spinal cord samples were post-fixed in 4% PFA at 4 °C overnight and cryoprotected with 30% sucrose solution before snap-frozen using dry ice. Samples were sectioned into 10 um slices with a Leica CM1950 cryostat (Leica Biosystems Nussloch GmbH, Germany). Cryosections were incubated in blocking buffer containing 1× PBS, 5% normal goat serum (Cat#: 16210064, Thermofisher Scientific) and 0.3% Triton™ X-100 (Cat#: T8787, Sigma-Aldrich) at room temperature for 1 hour, and then incubated with primary antibodies [anti-MBP (1:400, Cat#: NB600-717SS, Novus), anti-NF200 (1:400, Cat#: N4142, Sigma-Aldrich), anti-GFAP (1:400, Cat#: E4L7M, CST), anti-CSPG (1:400, Cat#: C8035, Sigma-Aldrich), anti-Iba1 (1:300, Cat#: ab178846, Abcam)] at 4 °C overnight. Sections were rinsed three times in 1×PBS solution before incubated with fluorochrome-conjugated secondary antibodies [Alexa Fluor® 488 goat anti-mouse/rabbit antibody (1:400, Cat#: ab150113/ab150077, Abcam), Alexa Fluor® 555 goat anti-rabbit antibody (1:400, Cat#: ab150078, Abcam)] at room temperature for 1 hour. After being rinsed thrice, sections were counterstained with DAPI-containing mounting medium to visualize cell nuclei and imaged using a Nikon inverted microscope (ECLIPSE Ti2, Nikon Corporation, Japan).

**Fecal DNA Extraction and 16S rRNA Gene Sequencing**

Fecal samples were collected on days 1, 3, and 14 after ICH. Bacterial DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (Code No. 51604, Qiagen) according to the manufacturer's instructions. Real-time quantitative polymerase chain reaction (RT-qPCR) was conducted for amplification of the 16S rRNA gene V4 hypervariable region with barcoded primers, forward primer (5′-GTGYYCAMCCGCGGCTAA-3′) and reverse primer (5′-GGACTACNVGGGTWTCTAAT-3′). The PCR amplicons were mixed and sequenced with an Illumina MiSeq platform. The preprocessing of the raw sequences was performed using the BIPES protocol. A Quantitative Insights into Microbial Ecology 2 (QIIME2) workflow script was used to control the sequence qualification. Based on the determined sequence frequency, the operational taxonomic unit (OTU) clustering was performed with Usearch algorithm. BIOM files were generated using a QIIME2 workflow script, pick_closed_reference_otus.py. Alpha diversity was represented by the Shannon index, which indicated the abundance and distribution evenness of bacterial species. Beta diversity was estimated using the weighted UniFrac distances method. The smaller weighted UniFrac distances meant the higher similarity. Principal coordinates
analysis (PCoA) was a dimensionality reduction method performed to visualize the relationship between samples based on the distance matrix. Linear discriminant analysis effect size (LEfSe) was an algorithm for high-dimensional biomarker discovery which was used to determine the discriminative characteristics between different groups. The linear discriminant analysis (LDA) score was applied to analyze the effect size of each differential abundance features. Microbial taxa with LDA score $\geq 3$ were listed on the taxonomy bar plots and cladogram.

**Total RNA Extraction And Relative Expression Levels Determine**

Total RNA of the brain and colon tissue was extracted using Trizol reagent (Code No. 9108, TaKaRa RNAiso Plus) following the manufacturer's guidelines. The reverse transcription reaction was performed by using PrimeScript™ RT reagent kit (Code No. RR037Q, TaKaRa) to generate cDNA, which was applied to subsequent quantitative PCR reaction using SYBR$^R$ Premix Ex Taq$^TM$ (Code No. RR820L, TaKaRa) on an Applied Biosystems ViiA™ 7 Real-Time PCR system (Thermo Fisher Scientific, USA). The relative mRNA levels of target genes were calculated by the $2^{-\Delta\Delta Ct}$ method and normalized to the expression level of $\beta$-actin.

**Gut Microbiota Depletion And Fecal Microbiota Transplantation**

Fecal samples were collected from the ICH + Vehicle group and ICH + OMT group at 3 days after ICH, then homogenized with sterile PBS (200 ul per 0.2 g feces) and centrifuged to obtain fecal suspension. To deplete gut microbiota, antibiotics mix (ampicillin 20 mg/ml, metronidazole 20 mg/ml, neomycin 20 mg/ml, and vancomycin 10 mg/ml, Sigma-Aldrich) were dissolved in sterile water and administrated by oral gavage for 1 week. The fecal microbiota transplantation (FMT) was conducted by daily oral gavage with 200 ul fecal suspension for 3 days after ICH surgery [21].

**Serum Inflammatory Cytokines And Lipopolysaccharide Measurement**

Venous blood samples were centrifuged for 15 minutes at 4 °C after natural coagulation. serum samples were transferred into a new tube for subsequent analysis. The serum levels of mouse interleukin-1$\beta$ (IL-1$\beta$, Cat#: MM-0040M1), interleukin-6 (IL-6, Cat#: MM-0163M1), tumor necrosis factor-alpha (TNF- $\alpha$, Cat#: MM-0825M1) and lipopolysaccharide (LPS, Cat#: MM-0634M1) were measured using commercial ELISA kits (Meimian, China), according to manufacturer’s protocol.

**Statistical Analysis**

All data were expressed as the mean ± standard deviation (mean ± SD). Statistical analysis was performed using the SPSS software version 20.0 (SPSS, Chicago, IL, USA). For normal distribution variables, the student's $t$ test was used to compare the means of two groups. Comparisons among
multiple groups were analyzed using one-way analysis of variance (ANOVA) with a *post hoc* analysis with the Bonferroni method. Continuous neurological function scores were analyzed by two-way ANOVA adjusted by Tukey’s *post hoc* test. Regarding non-normal distribution variables, the Kruskal-Wallis H test and Mann-Whitney U test were conducted for statistical analysis between different groups. Correlation analysis between bacterial species and inflammatory markers was evaluated by Spearman’s rank correlation. GraphPad Prism Software version 6.0 (GraphPad Prism, San Diego, CA, USA) was applied to visualize analysis results. The *P* value < 0.05 was considered statistically significant. Sham group versus ICH + Vehicle group, *P* < 0.05, **P** < 0.01, ***P*** < 0.001, ICH + Vehicle group versus ICH + OMT group, #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001.

**Results**

**OMT alleviated the neurological deficits after ICH**

OMT by oral administration for 3 days reduced hematoma volume and brain water content (BWC, in ipsilateral hemilateral) compared with that of vehicle-treated mice after ICH (Fig. 1B, C). Then we investigated inflammation after ICH by qPCR and found that NLRP3 inflammasome complex (Nlrp3, Asc and Caspase-1) and pro-inflammatory cytokines (Il-1β, Il-6, Tnf-α, and Nos2) mRNA levels were significantly elevated in peri-hematoma region on days 3 and 14, which were down-regulated by OMT treatment (Additional file 1: Fig. S1A, B).

The neurological function was continuously measured for 2 weeks after ICH via the corner turn test and mNSS. The mNSS test showed that the neurological function was markedly impaired in the ICH + Vehicle group, compared with sham control, among which the scores reached the peak on day 3 and thereafter gradually declined on days 7 and 14 after the ICH operation. Instead, OMT partial alleviated ICH-induced neurological deficits (Fig. 1D). Similarly, OMT administration significantly reduced the proportion of right turn after ICH (Fig. 1E). Taken together, these results suggested that OMT could suppress hematoma expansion and brain edema development, inhibit neuroinflammation, alleviate neurological deficits.

**Omt Alleviated White Matter Injury And The Formation Of Glial Scar After Ich**

The integrity of white matter was assessed by immunostaining with MBP (a marker of myelin sheath) and NF200 (a marker of axons) (Additional file 2: Fig. S2A). The mean fluorescence intensity (MFI) ratio of MBP/NF200 was used to quantitatively analyze these changes. Our results demonstrated that both MBP and NF200 were markedly destroyed by ICH, resulting in a sharp decrease of the MFI of MBP and NF200. The MFI of them was significantly increased in the OMT-treated group (Fig. 2A, Additional file 2: Fig. S2A). These findings suggested that OMT attenuated the WMI around the hematoma region.
Subsequently, we detected the expression of MBP and NF200 in the CST region of cervical enlargement to further clarify secondary WMI. The results showed that the expression of NF200 was dramatically reduced after ICH, consistent with that in the peri-hematoma region, which indicated axonal Wallerian degeneration may extended to the CST region of cervical enlargement. The MFI of NF200 was significantly increased after OMT administration (Fig. 2B). Nevertheless, the MFI of MBP in the CST region was not significantly different in ICH mice with or without OMT treatment (Fig. 2B). This may attribute to the heterogeneous pathogenic processes of the axonal degeneration and demyelination. Our results indicated that OMT attenuated both the proximal and the distal WMI at 14 days after ICH.

The formation of glial scar is regarded as another injury indicator after ICH. The chondroitin sulfate proteoglycan (CSPG) is an extracellular matrix compound, derived from reactive astrocytes, which is the major inhibitor of axonal growth. A mass of activated astrocytes, detected by the colocalization of GFAP and CSPG, aggregated at the peri-hematoma region after ICH (Fig. 2C, Additional file 2: Fig. S2B, C). The same phenomenon was discovered in the CST region (Fig. 2D, Additional file 2: Fig. S2D), suggesting similar pathogenic changes emerged in the distal WM of the spinal cord. While the number of GFAP and CSPG double-labeled astrocytes was significantly reduced in the OMT-treated mice compared with their counterpart in the ICH + Vehicle group (Fig. 2C, D; Additional file 2: Fig. S2B-D). Besides, ICH induced more activated microglia to aggregate in the CST region in response to axon injury and myelin destruction. While OMT treatment significantly attenuated glial scar formation and microgliosis in the CST region (Fig. 2C, Additional file 2: Fig. S2B-E). Our results indicated that OMT significantly inhibited the formation of glial scar and microgliosis at both the proximal and distal WM after ICH.

**Omt Modulated The Gut Dysbiosis Induced By Ich**

To investigate the composition of gut microbiota after ICH and the effect of OMT on this alteration, 16S rRNA gene sequencing was performed. Microbial profiling depicted that in the acute phase after ICH, the α-diversity, calculated by the Shannon index, manifested a significant decline in both the vehicle and OMT treated groups compared with the sham group (Fig. 3A, Additional file 3: Fig. S3A). Additionally, the β-diversity, reflecting the differences between microbial communities, estimated by PCoA analysis based on the weighted UniFrac distance, displayed that samples from the ICH + Vehicle group were clearly separated from those from the Sham group on days 1 and 3 after ICH. And this phenomenon was only observed at 3 days after ICH between the ICH + Vehicle group and the ICH + OMT group (Fig. 3B, Additional file 3: Fig. S3B). Microbial population analysis revealed that ICH remarkably altered the gut microbial composition, and OMT treatment modulated the composition of gut microbiota at the phyla and the genus levels. LEfSe analysis Showed that at 1 day after ICH, the relative abundance of Rikenellaceae and S24_7 was significantly higher but those of Bacteroides, Parabacteroides, Coprococcus, and Proteobacteria were significantly lower in the ICH group compared with the Sham group. While OMT significantly increased the relative abundance of Bacteroides, Parabacteroides, and Proteobacteria and decreased those of Rikenellaceae after ICH (Additional file 3: Fig. S3C-F). At 3 days after ICH, members from Bacteroidetes, Rikenellaceae, and S24-7 were excessively overgrown, while the
relative abundances of *Lachnospiraceae*, *Coprococcus*, *Ruminococcaceae*, and *Oscillospira* which were mostly defined as butyrate-producing bacteria, and other microbial species from *Firmicutes*, *Proteobacteria*, *Desulfovibrionaceae*, and *Desulfovibrio*, were all significantly decreased after ICH, and those were increased under OMT administration but with no statistical significance (except *Proteobacteria*), including the ratio of F/B. On the other hand, *Bacteroides*, *Parabacteroides*, *Helicobacter*, and *Akkermansia* were more enriched in the ICH + OMT group compared with the ICH + Vehicle group (Fig. 3C, Additional file 4: Fig. S4A-C).

In the chronic phase after ICH, there was still a significant decline in the species richness in the ICH + Vehicle group as shown by the Shannon index (Fig. 4A). PCoA analysis revealed that samples from different groups were clearly separated from each other, suggesting the composition of gut microbiota was discriminated between different groups (Fig. 4B). Using LEfSe analysis, we determined that *Bacteroidetes, S24-7, Paraprevotellaceae, Prevotella*, and *Akkermansia* become the predominated bacteria in the ICH + Vehicle group, but the relative abundance of above-mentioned species (except *Akkermansia*) was significantly decreased in the ICH + OMT group (Fig. 4C, Additional file 4: Fig. S4D-F). Meanwhile, ICH induced a lower abundance of *Firmicutes*, *[Ruminococcus]*, and *Turicibacter*, and a lower F/B ratio. In contrast, *Bacteroides, Parabacteroides, Ruminococcus*, and *Turicibacter* significantly enriched in the ICH + OMT group compared to the ICH + Vehicle group (Fig. 4C, Additional file 4: Fig. S4D-F). All these results indicated that ICH induced obvious gut dysbiosis, and OMT could modulate gut microbiota.

**Omt Ameliorated Intestinal Barrier Disruption And Gut-derived Endotoxemia After Ich**

To determine intestinal barrier function, we detected the mRNA expression of intestinal tight junction proteins. The mRNA levels of target genes including ZO-1, Occludin, and Claudin-4 were significantly decreased on both days 3 and 14 after ICH. Treatment with OMT significantly up-regulated the expression levels of tight junction proteins (Fig. 5A, B). We further evaluated the permeability of the intestinal barrier on days 3 and 14 after ICH. The concentration of FD4 in the serum was increased after ICH at both time points, those level was decreased under OMT administration (Fig. 5C, D). Taken together, ICH caused intestinal barrier dysfunction with tight junction proteins degradation and permeability increasing, while the application of OMT dramatically reversed the disruption of the intestinal barrier.

The serum concentrations of LPS and several inflammatory factors including TNF-α, IL-6, and IL-1β were obviously increased, which were reversed by OMT (Fig. 5E, F). In summary, OMT attenuated the gut-derived endotoxemia after ICH.

**The Correlation Between Significantly Differential Microbiome Taxa And Injury-associated Indexes After Ich**
In order to investigate the correlation between the relative abundance of microbial species and ICH-induced injury associated indexes, Spearman's rank correlation was conducted. At 3 days after ICH, members from the *Bacteroidetes* phylum, including *Rikenellaceae* and *S24-7*, were significantly positively correlated with several inflammatory markers (i.e., IL-1β, IL-6, TNF-α, and LPS), serum concentration of FD4 and behavioral scores, but negatively correlated with the mRNA levels of intestinal tight junction proteins (Tjs). While there was a negative correlation between microbial species from *Firmicutes* and *Proteobacteria* phylum, specially *Lachnospiraceae*, Desulfovibrionaceae, *Ruminococcaceae*, *Oscillospira*, and *Desulfovibrio* and those above markers except intestinal Tjs expression (Fig. 6A). At 14 days after ICH, microbial species from *Bacteroidetes* phylum, such as *Paraprevotellaceae*, *Prevotellaceae S24-7*, and *Prevotella*, still had a positive correlation with those markers, but negative correlation with Tjs expression. While a negative correlation was found between *Firmicutes*, *Ruminococcus*, and *[Ruminococcus]* and those markers, microbial taxa from *Firmicutes*, Turicibacteraceae, Turicibacter, *Ruminococcus*, and *[Ruminococcus]* had a significantly positive correlation with Tjs expression (Fig. 6B). To sum up, the relative abundances of some microbial species were significantly associated with ICH-induced pathological changes. However, whether a causal relationship between them exists require further investigation.

**Gut Microbiota Partially Mediated The Therapeutic Effects Of Omt On Ich**

We further conducted FMT to detect the role of the gut microbiota in the pathogenic processes of ICH (Fig. 7A). At 3 days after ICH, recolonization with ICH + OMT-fecal microbiota significantly reduced mNSS scores and right turn bias, compared with ICH-fecal microbiota (Fig. 7B). For intestinal barrier permeability detection, mice recolonized with ICH-fecal microbiota exhibited higher serum fluorescein concentrations than those recolonized with ICH + OMT-fecal microbiota, suggesting lower permeability of intestinal barrier after ICH + OMT-fecal microbiota recolonization (Fig. 7C). In addition, the mRNA levels of tight junction proteins (ZO-1) were significantly decreased in the FMT-ICH + OMT group compared with the FMT-ICH + Vehicle group (Fig. 7D).

Furthermore, 16S rRNA sequencing was performed to reveal the gut microbial composition in recipient mice who received FMT for 3 days. The α-diversity in the FMT-ICH + Vehicle group was significantly lower than those in the FMT-ICH + OMT group (Fig. 7E). As the PCoA analysis showed, the distribution of samples from the FMT-ICH + Vehicle group was clearly separated from those from the FMT-ICH + OMT group, and the difference in PC1 axis reached significant(Fig. 7F). Population analysis demonstrated that the FMT-ICH + Vehicle group displayed a higher abundance of *Proteobacteria* and a lower abundance of *Bacteroidetes* than the FMT-ICH + OMT group at the phylum level (Additional file 4: Fig. S4G). The abundance of the predominant seven genus was different between them at the genus level (Additional file 4: Fig. S4H). LEfSe results revealed that members from the *Proteobacteria* phylum, such as *Coriobacteriaceae*, *Enterobacteriaceae*, *Escherichia*, *Adlercreutzia*, mitochondria, *Desulfovibrio*, *Pseudomonas*, *Staphylococcus*, and *Clostridiales*, were enriched in the FMT-ICH + Vehicle group,
compared to those in the FMT-ICH + OMT group (Fig. 7G, Additional file 4: Fig. S4I). While the relative abundance of *Bacteroidetes*, *Bacteroidaceae*, *Bacteroides*, *Parabacteroides*, *Proteus*, and *Myroides* in the FMT-ICH + Vehicle group were significantly lower than those in the FMT-ICH + OMT group (Fig. 7G, Additional file 4: Fig. S4I). Above all, the therapeutic effects of OMT on ICH-induced neurological deficits and intestinal barrier dysfunction were partially mediated by gut microbiota. However, the mechanism underlying requires a more thoroughly designed study to investigate.

Discussion

WMI induced by ICH plays an important role in the neurological function outcome[22]. In this study, it was shown that ICH can not only cause extensive WMI in the primary lesion but also induce persistent secondary white matter fiber tracts injury at the cervical enlargement, followed by gut dysbiosis, intestinal barrier dysfunction, and systemic inflammation. Oral administration with OMT significantly alleviated ICH-induced WMI in the hematoma region and distal spinal segment, modulated gut microbial composition, and ameliorated intestinal barrier dysfunction. FMT experiment confirmed that gut microbiota was involved in this pathophysiological process after ICH (Additional file 5: Fig. S5).

OMT was extensively utilized for several diseases, such as cirrhosis, chronic heart failure, colitis, and so on[23–26]. It was reported that OMT displayed therapeutic effects on several neurological disorders, such as ischemic stroke, spinal cord injury, and so on. Liu et al. found that OMT reduced infarct volume and downregulated the expression of NF-κB after ischemic stroke[17]. In an experimental SCI model, OMT promoted neuromotor function recovery by alleviating axonal injury and promoting axonal regeneration[27]. Baseline hematoma volume and subsequently hematoma expansion are established prognostic factors of both mortality and functional outcome after ICH[28, 29]. Besides, secondary brain injury caused by mass effect and release of blood components, such as edema and inflammation, also plays an important role in the neurological function recovery after ICH[30]. Current study revealed that OMT administration (120 mg/kg) conferred neuroprotective effects on ICH by reducing hematoma volume and inhibiting the formation of edema. Accumulating studies demonstrated that OMT displayed remarkably anti-inflammation and immunomodulation effects. OMT exerted protections against rheumatoid arthritis and ulcerative colitis, by inhibiting inflammation and modulating T lymphocyte immune homeostasis[25, 31]. OMT alleviated ischemic cerebral injury by inhibiting the TLR4/NF-κB signaling pathway in an experimental stroke model[17]. The nucleotide-binding oligomerization domain-, leucine-rich repeat and pyrin domain-containing 3 (NLRP3) inflammasome is a critical receptor and sensor of the innate immune system that aggravated inflammatory response and brain edema progression after ICH, and selectively inhibiting NLRP3 inflammasome activation attenuated ICH-induced brain injury and neurological function deficits[33–35]. However, little is known about the effects of OMT on NLRP3 inflammasome activation. In this study, we found that OMT significantly downregulated NLRP3 inflammasome complex mRNA levels at 3 and 14 days after ICH. Additionally, OMT also reduced the mRNA level of other pro-inflammatory cytokines after ICH, suggesting OMT could effectively inhibit neuroinflammation in the brain. However, the underlying mechanism of the inhibition effect of OMT on NLRP3 inflammasome and neuroinflammation still needs to be further verified.
WMI is an important predicted factor for poor outcomes after ICH[36]. The severity of WMI is closely related to neuromotor dysfunction in ICH patients[7]. In present study, OMT significantly alleviated WMI in the peri-hematoma region at 14 days after ICH. The less extensive WMI in the primary lesion may be account for better neurological function recovery after ICH. Besides, ICH-induced secondary remote white matter fiber tracts (such as CST) injury also plays a pivotal role in the functional outcome. CST is the primary descending motor projection that connects the primary motor cortex and the spinal cord[37]. In stroke patients, the integrity of the CST was a determining factor for the proportional recovery of motor impairment[38]. Quantitative tractography revealed that there were more reconstructed CST fiber pathways in ICH patients with favorable outcomes[39]. Our study revealed that treatment with OMT significantly alleviated ICH-induced CST axon injury and demyelination at cervical enlargement, which is perhaps due to the protection of OMT against WMI in the primary lesion, or it’s inhibition of astrocytes/microglia activation and inflammatory response.. Additionally, detecting the immunophenotype of glial cells, we found that ICH activated more microglia and more astrocytes co-labeled with GFAP and CSPG in the CST region of the cervical enlargement, suggesting that ICH caused CST destruction in the cervical enlargement, which subsequently induced microglia and astrocytes activation, while OMT significantly inhibited their activation and inflammatory response in the CST after ICH. Prolonged activation of microglia induced by myelin debris has long been implicated in aggravating inflammatory action by secreting pro-inflammatory cytokines[40, 41]. Whereas Cunha et al. revealed that the pro-inflammatory phagocytic phenotype of microglia is essential for myelin debris clearance and remyelination[42]. In our study, microglia activation mitigated by OMT treatment after ICH perhaps due to the directed inhibition effect of OMT or less severe CST injury. Furthermore, OMT significantly alleviated ICH-induced neurological deficits both in the acute phase and the chronic phase. Perhaps slighter primary WMI in the peri-hematoma region and secondary CST injury account for better neurobehavioral performance after OMT treatment.

Gut microbiota is involved in the development and progression of central nervous system diseases, such as ischemic stroke, through the microbiota-gut-brain axis[43]. Recolonized with stroke-induced dysbiotic microbiome from special pathogen-free mice, germ-free mice developed a larger infarct volume after distal middle cerebral artery occlusion[44]. Additionally, transplantation with youthful gut microbiota or short chain fatty acids-producing bacteria signicantly alleviated neurological impairment after stroke in aged mice[45]. Moreover, a recent study by Xu et al. revealed that ischemic stroke induced significantly Enterobacteriaceae outgrowth and intestinal barrier disruption, while inhibiting Enterobacteriaceae expansion alleviated brain injury after stroke[46]. However, the role of gut microbiota in the ICH outcome is still poorly understood. Interestingly, when administrated orally, OMT will be converted into the more absorbable metabolite matrine (MT) by gut bacteria in the gastrointestinal tract[47]. 16S rRNA sequencing revealed that oral administration with MT dramatically sharpened gut microbial community, resulting in more beneficial commensal genera, such as Ruminoclostridium, Lachnospiraceae, and Ruminococcaceae[48]. In an experimental ulcerative colitis model, MT attenuated colitis severity by modulating microbiota and suppressing inflammatory signal transduction[49]. Whether OMT shaped gut microbial composition remained largely unknown. Our results revealed that ICH caused persistent
microbiome disturbances at consecutive time courses. OMT significantly modulated gut microbial composition after ICH. Moreover, correlation analysis revealed that bacteria richness were significantly correlated with inflammation, intestinal barrier permeability, and neurological deficits after ICH. However, little is known about the precise mechanism by which differential bacteria affected the pathological process of ICH.

Intestinal injury, especially gastrointestinal bleeding, is a common critical complication in ICH patients and an important risk factor for poor outcomes [50, 51]. In an animal model, ICH induced rapid and persistent impairment of intestinal barrier function and inflammation [52]. Another study by Yu et al. also found that gastrointestinal dysfunction, with reducing small intestine motility, increasing intestinal barrier permeability, and disrupting intestinal integrity, occurred after ICH [14]. In experimental pancreatitis, ulcerative colitis, and hepatic cirrhosis models, OMT protected the intestinal barrier by inhibiting inflammation [23, 53, 54]. In this study, ICH induced significant disruption of intestinal barrier integrity and increased intestinal permeability, inflammatory cytokines, and LPS levels in the bloodstream. OMT significantly reversed these pathological changes induced by ICH and significantly alleviated the impairment of intestinal function and circulating inflammatory response.

Although our study has provided evidence that oral administration of OMT significantly alleviated ICH-induced neurological deficits, gut dysbiosis, intestinal barrier dysfunction, and systemic inflammation, and FMT confirmed that gut microbiota plays an important role in these pathological processes, there were several limitations in the present study. Firstly, medicine metabolism by bacteria in the intestine usually affects their pharmacokinetics and efficacy [55–57]. Further research investigating the role of OMT metabolite by the microbiome in ICH is warranted. Secondly, gut microbiota-derived metabolites participated in the pathophysiology of several diseases [58, 59]. The impact of OMT on intestinal metabolites derived from the microbiome requires further study. Thirdly, although the role of gut microbiota in ICH was verified by the FMT experiment, the underlying mechanism still needs further study to elucidate.

**Conclusion**

In conclusion, our study is the first time to reveal that OMT treatment effectively reduced WMI both at the striatum and distal CST region of the cervical enlargement, improved ICH-caused gut dysbiosis, promoted the intestinal barrier reconstruction, and inhibited the systemic inflammatory response. The role of intestinal flora in the effects of OMT on alleviating WMI induced by ICH still needs to be further studied.

**Abbreviations**

ICH, intracerebral hemorrhage; WMI, white matter injury; CST, corticospinal tract; CNS, central nervous system; OMT, Oxymatrine; FMT, fecal microbiota transplantation; NLRP3, nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing 3; NF-κB, nuclear factor kappa B; TLR4, toll-like receptors 4; PBS, phosphate buffer saline; mNSS, Modified Neurological Severity Score;
BWC, brain water content; FD4, 4 kDa fluorescein isothiocyanate (FITC)-dextran; PFA, paraformaldehyde; MBP, myelin basic protein; NF200, Neurofilament 200; GFAP, glial fibrillary acidic protein; CSPG, chondroitin sulfate proteoglycans; Iba1, ionized calcium-binding adapter molecule 1; PCoA, principal coordinate analysis; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; CTT, Corner turn test; PCR, Polymerase Chain Reaction; IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-, tumor necrosis factor-alpha; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; Tjs, tight junction proteins; SCI, spinal cord injury; MT, matrine.

Declarations

Ethical Approval and Consent to participate

The experimental protocol was approved by the Institutional Ethics Committee of Zhujiang Hospital of Southern Medical University (LAEC-2020-227).

Consent for publication

Not applicable.

Availability of data and materials

Data and materials will be shared upon reasonable request.

Competing interests

The authors declare no competing interests.

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Authors’ contributions

The work presented here was carried out in collaboration among all authors. Haitao Sun conceived and designed the study. Hongwei Zhou, Wen Yuan, and Junshan Liu provided support of essential lab resources for this study. Jing Li conducted this study and drafted this manuscript; Jianhao Liang, Meiqin Zeng, Kaijian Sun, Yunhao Luo, Huaping Zheng, and Feng Li analyzed the data. Haitao Sun revised the manuscript. All authors reviewed and approved the final manuscript.

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Figures
OMT alleviated the neurological deficits after ICH. A The experimental protocol for OMT treatment after ICH. B, C hematoma volume (B, n=6-7/group) and brain water content (%) (C, n=4-5/group) at 3 days after ICH. D, E mNSS score (D, n=9-10/group) and corner turn test (E, n=9-10/group) at days 1, 3, 7, 14 after ICH. Sham group versus ICH+Vehicle group, *P 0.05, **P 0.01, ***P 0.001, ICH+Vehicle group versus ICH+OMT group, #P 0.05, ##P 0.01, ###P 0.001.
Figure 2

OMT alleviated white matter injury and the formation of glial scar after ICH. **A, B** Immunodetection for MBP and NF200 in the peri-hematoma region (A) and distal CST injury region of cervical enlargement (B) at 14 days after ICH, and the result of quantitative analysis of their mean fluorescence intensity (MFI). The immunofluorescence amplification of representative images in the third column (A) was 10×20, Scale bar=100 μm. The images in the fourth column are 10×40, Scale bar=50 μm. \( n=5-6/\text{group}. \) The
The expression of CSPG and GFAP in the peri-hematoma region (C) and distal CST injury region of cervical enlargement (D) at 14 days after ICH. The magnification of the immunofluorescence field (C) was 10×40, Scale bar=50 μm. The magnification of the immunofluorescence field (C) was 10×40, Scale bar=50 μm. The immunofluorescence amplification of images (D) was 10×20, Scale bar=100 μm. The images within the white box in the last column are 10×40, Scale bar=50 μm. Sham group versus ICH+Vehicle group, *P 0.05, **P 0.01, ***P 0.001, ICH + Vehicle group versus ICH+OMT group, #P 0.05, ##P 0.01, ###P 0.001.
OMT modulated the gut dysbiosis at 3 days after ICH. A, B Comparison of α-diversity (A, Shannon index), and β-diversity (B, PCoA analysis based on weighted UniFrac distance) among three groups at 3 days after ICH. C The distribution bar plot based on the LEfSe analysis (LDA score (log 10) 3) and the richness of significantly differential taxa among three groups at 3 days after ICH.  n=9-10/group. Sham group
versus ICH+Vehicle group, *P 0.05, **P 0.01, ***P 0.001, ICH+Vehicle group versus ICH+OMT group, #P 0.05, ##P 0.01, ###P 0.001.

**Figure 4**

OMT modulated the gut dysbiosis at 14 days after ICH. A, B Comparison of α-diversity (A, Shannon index), and β-diversity (B, PCoA analysis based on weighted UniFrac distance) among three groups at 14 days
after ICH. C The distribution bar plot based on the LEfSe analysis (LDA score (log 10) 3) and the richness of significantly differential taxa among three groups at 14 days after ICH. n=9-10/group. Sham group versus ICH+Vehicle group, *P 0.05, **P 0.01, ***P 0.001, ICH+Vehicle group versus ICH+OMT group, #P 0.05, ##P 0.01, ###P 0.001.

Figure 5
OMT ameliorated intestinal barrier disruption and gut-derived endotoxemia after ICH. **A-D** The mRNA levels of tight junction proteins (ZO-1, Occludin, and Claudin-4. **A**, \(n=8\)/group; **B**, \(n=8\)/group) in colon tissues and serum FD4 concentrations (**C**, \(n=5\)/group; **D**, \(n=6\)/group) on day 3 and 14 after ICH. **E-F** Serum LPS and inflammatory factors (IL-1β, IL-6, and TNF-α) concentrations at 3d (**E**, \(n=6\)/group) and 14d (**F**, \(n=6\)/group) after ICH. Sham group versus ICH+Vehicle group, \(*P \leq 0.05, **P \leq 0.01, ***P \leq 0.001, ICH+Vehicle group versus ICH+OMT group, \#P \leq 0.05, ##P \leq 0.01, ###P \leq 0.001.

**Figure 6**

The correlation between significantly differential microbiome taxa and injury-associated indexes after ICH. **A** The heatmap indicated the correlation between microbial species and injury-associated indexes on days 3 (left) and 14 (right) after ICH. Red and green cells indicated positive and negative correlations, respectively. \(*P \leq 0.05, **P \leq 0.01, ***P \leq 0.001.\)
Figure 7

Gut microbiota mediated the therapeutic effects of OMT on ICH. **A** The experimental protocol for FMT after ICH. **B** mNSS score (n=10/group) and corner turn test (n=9-10/group) on days 1 and 3 after ICH. **C, D** Serum FD4 concentrations (C, n=6/group) and the mRNA levels of tight junction proteins (D, ZO-1, Occludin, and Claudin-4, n=8/group) in colon tissues on day 3 after ICH. **E, F** Comparison of -diversity (E, Shannon index), β-diversity (F, PCoA analysis based on weighted UniFrac distance) at 3 days after ICH. **G**
The distribution bar plot based on the LEfSe analysis (LDA score (log 10) 3), and the richness of significantly differential taxa between the two groups at 3 days after ICH. n=9-10/group. Sham group versus ICH+Vehicle group, *$P$ 0.05, **$P$ 0.01, ***$P$ 0.001, ICH+Vehicle group versus ICH+OMT group, #$P$ 0.05, ##$P$ 0.01, ###$P$ 0.001.

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