A Network Pharmacology-Based Analysis of the Protective Mechanism of Miao Medicine Xuemaitong Capsule against Secondary Brain Damage in the Ischemic area Surrounding Intracerebral Hemorrhage

**Running title:** Protective mechanism of Xuemaitong Capsule against ICH

Bo Zhang 1,2, #, *, Zhengyan Zeng 3, #, Haijun Wu 2, #

1 Neurosurgery Department, Huiya Hospital of the First Affiliated Hospital, Sun Yat-Sen University, Huizhou 516081, P. R. China

2 Neurosurgery Department, the First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine, Guiyang 550006, P. R. China

3 Graduate School, Guizhou University of Traditional Chinese Medicine, Guiyang 550025, P. R. China

# Bo Zhang & Zhengyan Zeng & Haijun Wu are co-first authors

* Correspondence to: Bo Zhang Neurosurgery Department, Huiya Hospital of the First Affiliated Hospital, Sun Yat-Sen University, No. 186, Northern Zhongxing Road, Dayawan District, Huizhou 516081, Guangdong Province, P. R. China; Neurosurgery Department, the First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine, No. 71, Baoshan North Road, Yunyan District, Guiyang 550006, Guizhou Province, P. R. China

E-mail: zhbbls@163.com

Tel: +86-0752-6516990-8160


**Abbreviation**

ANOVA: Analysis of variance
BBB: Blood-brain barrier
CASP3: Caspase-3
cDNA: Complementary DNA
DAVID: Database for Annotation, Visualization and Integrated Discovery
DISC: Death inducing signaling complex
ERK: Extracellular signal-regulated kinase
FADD: Fas-associated Death Domain
FJB: Fluoro-Jade B
ICH: Intracerebral hemorrhage
HE: Hematoxylin-Eosin
JNK: Jun amino-terminal kinase
KEGG: Kyoto Encyclopedia of Genes and Genomes
PBS: Phosphate buffer saline
PPI: Protein-protein interaction
RT-qPCR: Reverse Transcription Quantitative Real-Time PCR
RIP1: Receptor interacting protein 1
TNF: Tumor necrosis factor
TNFR1: TNF receptor 1
TRAF2: TNF receptor associated factor 2
TCM: Traditional Chinese medicine
TRADD: TNFRSF1A associated via death domain
XMT: Xuemaitong
ABSTRACT

Intracerebral hemorrhage (ICH) is a devastating disease with the high mortality. The poor outcome of ICH is partially due to combination of various secondary insults, including ischemic area. Xuemaitong capsule (XMT), a kind of traditional Chinese medicine, has been applied to clinic practice. The purpose of this study is to explore the mechanism of XMT in alleviating secondary damage in ischemic area after ICH. We screened XMT target, compound components and ICH-related targets using network pharmacology, cluster analysis, and enrichment analysis. We found that TNF signaling pathway might be the key signaling pathway for XMT treatment of ICH. ICH rat model was established, as demonstrated by poor neurological score. In the ICH rats, Western blot analysis and immunofluorescence indicated the upregulated expression of TNFR1, MAPK, NF-κB and caspase-3. Importantly, administration of XMT alleviated inflammation, edema and increased perfusion in ischemic area, while the expression of TNFR1, MAPK, NF-κB and CASP3 was decreased. Besides, Fluoro-Jade B and TUNEL staining revealed that XMT application also inhibited apoptosis and degradation of ischemic area neurons. In conclusion, these evidence elucidates that XMT alleviates neuron apoptosis, ischemic area inflammation, edema, and perfusion through TNFR1-mediated CASP3/NF-κB/MAPK axis.

Keywords: Xuemaitong; Intracerebral hemorrhage; TNF signaling pathway; Ischemic area; Neurons; Apoptosis; Edema; Inflammation; Hypoperfusion
SIGNIFICANCE STATEMENT

TNF is the key signaling pathway of XMT to intervention during ICH. 14 key targets (ICAM1, IL6, TNF, CCL2, PTGS2, RELA, MMP9, EDN1, MAPK1, FOS, CASP3, JUN, IL1B, MAPK8) are retrieved from the database. XMT can inhibit neuron apoptosis in the ischemic area via regulating TNFR1/CASP3. XMT alleviates inflammation and edema through regulating TNFR1/NF-κB and TNFR1/MAPK signaling pathways. XMT alleviates hypoperfusion in the cerebral ischemic area through mediating TNFR1/MAPK/EDN1.
Introduction

Intracerebral hemorrhage (ICH), one of the common stroke with high morbidity and mortality globally, is characterized by the pathological aggregation of blood in patients’ brain (Giakoumettis et al., 2017; Zeng et al., 2018). Studies indicate that hypertension and cerebral amyloid angiopathy are the main risk factors for ICH (Passos et al., 2016). Not only structural damage but also secondary injury leads to poor prognosis in the peri-hemorrhagic region (Mittal and Lackamp, 2016). It is known that clinical recovery after stroke relies on the salvage of the ischemic area (Carrera et al., 2013), and the viable tissue near the injured ischemic core (Liu et al., 2010). Notably, application of traditional Chinese medicine (TCM) has been suggested for treatment of ICH (Chen, 2015). Therefore, it is potential to develop TCM for preventing secondary damage in the ischemic area after ICH.

Recent years witness the improvement and advancement of multi-target drugs or multi-component therapy. TCM has been suggested as a crucial resource for discovering multi-targets drugs (Wang et al., 2012). Network pharmacology, a novel discipline following the systems biology theory, is used to analyze the biological network. Also, it helps to select the nodes of interest and promote drug development in a more cost-effective way, which can be instructive for analysis of TCM (Hao da and Xiao, 2014). Of TCM, Xuemaitong (XMT) is a known Miao medicine and has been widely applied to clinical practice in China. It is composed of Cinnamomi Ramulus, Radix Salviae, Chuanxiong Rhizoma, Radix Puerariae, Gardeniae Fructus and Alisma Orientale (Sam.) Juz. According to previous reports, XMT can attenuate carotid atherosclerosis (Zhang et al., 2014). XMT granules have also been reported to improve blood rheology in rat atherosclerosis (Qi et al., 2005). In addition, many previous studies have indicated that components of XMT are beneficial for stroke therapy. For instance, a fermented Chinese formula Shuan-Tong-Ling, a fermented Chinese formulate which contains Radix Puerariae and Radix
Salviae, has been unfolded to ameliorate ischemic stroke through suppression of inflammation and apoptosis (Mei et al., 2017). Moreover, combined use of Radix Puerariae and Chuanxiong Rhizoma is widely applied for treatment of cerebrovascular diseases and has been implicated beneficial for alleviating cerebral ischemic stroke (Chen et al., 2019). Intriguingly, our database-based network analysis identified the tumor necrosis factor (TNF) signaling pathway as the key pathway for XMT treatment of ICH. TNF, a pro-inflammatory cytokine, contributes to mammalian immunity and cellular homeostasis while its aberrant expression is associated with many inflammatory disorders including stroke (Brenner et al., 2015). The progression of stroke is affected by the intricate relationship between the blood-brain barrier (BBB) and TNF (Pan and Kastin, 2007). A previous protein-protein interaction (PPI) network-based analysis also revealed the involvement of the TNF signaling pathway in stroke (Lv et al., 2020). Furthermore, a TCM prescription, Huang-Lian-Jie-Du Decoction, has 28 target proteins in stroke network, while it alleviates stroke via a pharmacological mechanism in part by mediating the TNF signaling pathway (Wang et al., 2019). However, little evidence elucidates the role of XMT in ICH. Therefore, herein we performed network pharmacology in this study to investigate whether XMT can be applied for ICH treatment. Based on the results of bioinformatic analysis, we established rat model of ICH and administered rats with XMT, while target gene expression and signaling pathway were detected.

**Materials and Methods**

**Ethics Statement**

The present study was approved by the Ethics Committee of the Animal of Guizhou Medical University. All animal experiments were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.
Composition Analysis of XMT Compounds

Through the TCMSP database (http://tcmsp.com/tcmsp.php) (screening conditions: OB ≥ 30%, DL ≥ 0.18), we analyzed the compound components of Cinnamomi Ramulus, Radix Salviae, Chuanxiong Rhizoma, Radix Puerariae, Gardeniae Fructus, Alisma Orientale (Sam.) Juz in XMT. The compound components of Euscaphis japonica (score cutoff ≥ 20, p value ≥ 0.05) were screened through the BATMAN database (http://bionet.ncpsb.org/batman-tcm/). A total of 65 compounds were retrieved, including 2 in Euscaphis japonica, 6 in Cinnamomi Ramulus, 34 in Radix Salviae, 6 in Chuanxiong Rhizoma, 3 in Radix Puerariae, 12 in Gardeniae Fructus, and 7 in Alisma Oriental (Sam.) Juz.

Target Analysis of XMT Compounds

TCMSP and BATMAN databases were used to retrieve the targets of compounds in XMT. The official gene symbols corresponding to the target protein were retrieved from the UniProtKB database (http://www.uniprot/) (The species condition was limited to "HOMA sapiens"). As a result, 189 targets were obtained, including 6 corresponding compounds in Euscaphis japonica, 37 corresponding compounds in Cinnamomi Ramulus, 68 corresponding compounds in Radix Salviae, 22 corresponding compounds in Chuanxiong Rhizoma, 46 corresponding compounds in Radix Puerariae, 160 corresponding compounds in Gardeniae Fructus, and 11 corresponding compounds in Alisma Orientale (Sam.) Juz.

Analysis of ICH-Related Targets

Through the CTD database (http://ctdbase.org/detail.go?ACC=c452899 & type=Chem) and GeneCards (https://www.genecards.org/) databases, the targets of ICH were retrieved and the
screening condition was set as: CTD: influence score \(\geq 30\). In total, 3046 targets were retrieved from the CTD database and 2489 from the GeneCards. Moreover, 971 ICH-related targets were obtained by screening the intersecting targets retrieved from the two databases.

**PPI Network**

Based on the STRING database (https://stringDb.org), the XMT compound targets and ICH-related target interaction network (The species condition was limited to "HOMA sapiens") were obtained.

**Construction of Target Interaction Networks**

Major networks were as follows: (1) ICH-related target network; (2) TCM-compound-target network; (3) key target of XMT treatment for ICH-compound-TCM network; (4) TCM-compound-TNF signal pathway target-TNF signal pathway network. All networks were drawn using the Cytoscape (http://cytoscape.org/, ver. 3.7.1). At the same time, we analyzed the results of the networks using the Cytoscape website, and selected the key targets for XMT treatment of ICH with the screening conditions set at BC \(\geq\) Avg (BC), CC \(\geq\) Avg (CC) and De \(\geq\) Avg (De).

**Cluster Analysis**

In large PPI networks, the tightly connected regions that may represent molecular complexes are defined as topological modules or clusters, which have pure network properties. The aggregation of nodes with similar or related functions in the same network is called functional module. Disease module is a set of network components that together destroy cellular function and then cause a specific disease phenotype. Because topological module, functional module and
disease module have the same meaning in the network, a function module is equivalent to a
topological module, and a disease module can be regarded as interference and destruction of a
functional module. Finally, we can perform cluster analysis to obtain the topological modules
through the Cycloscape plug-in MCODE.

**Enrichment Analysis**

The functional enrichment webpage on Database for Annotation, Visualization and Integrated
Discovery (DAVID) online (https://david.ncifcrf.gov/) was used for analysis of Kyoto Encyclopedia
of Genes and Genomes (KEGG) and the signal pathway of key target of XMT in the treatment of
ICH. The DAVID v6.8 comprises a full Knowledgebase update to the sixth version of our original
web-accessible programs and a comprehensive set of functional annotation tools. KEGG
(https://www.kegg.jp/ or https://www.genome.jp/kegg/) is used to interpret genome sequences and
other high-throughput data with three generic categories of systems information, genomic
information and chemical information, and human-specific category of health information.

**Animal Grouping and Administration Regimen**

A total of 126 adult male Sprague Dawley (SD) rats weighing 200 ± 10 g were purchased from
Guizhou Laboratory Animal Engineering Technology Center. The specific-pathogen-free animals
underwent adaptive feeding at 20-25°C under 45-50% relative humidity for 3-4 days (12-hour
light/dark cycles). The rats were randomly and equally divided into 7 groups in a blinded manner,
including sham-operated group, ICH group, ICH + vehicle group, ICH + XMT group (21, 42, 84
mg/kg) or ICH + Edaravone group (2 mg/kg). Edaravone, a drug known to alleviate ICH with
neuroprotective effect was taken as a positive control in this study (Miao et al., 2020). XMT dose
conversion between human and rats was as follows: the dose given to experimental animals (D2) =
human dose (D1) × 6.3 mg/kg. The adult dose was 2.34 g/70 kg/day; therefore D2 = 2.34 g ÷ 70 kg × 6.3 × 0.2 kg = 0.04 g/day). After ICH modeling, each rat was gavaged twice a day in the XMT treatment group. In each gavage, each rat was treated with 0.04 g/day. ICH rats in Edaravone group were injected intraperitoneally once a day while in Vehicle group were given an equal volume of saline. XMT, Edaravone and normal saline were administrated at 10 minutes after modeling. At 24 hours after ICH, 16 brain tissues in each group were selected for subsequent a series of experiments. The last 8 rats in each group were tested for brain edema and neurological function scoring 72 hours after ICH.

ICH Modeling

As previously described (Meng et al., 2018), ICH model was established in rats by injecting 100 μL of autogenous blood. The rats were anesthetized and placed in a stereotactic frame (ZH-Blue Star B brain stereotactic apparatus, Huaibei Zhenghua biological instrument equipment Co., LTD, Anhui, China). Next, the whole blood was collected via femoral artery puncture. The microinjector (Hamilton company, Nevada, USA) was inserted into bone hole (3 mm lateral to midline and 1 mm front to bregma, ventral side of cortex surface: 5.5 mm). First, 20 μL blood was injected into and maintained for 5 minutes and then 30 μL blood was injected into and maintained for 5 minutes. After the injection, the microinjector was slowly withdrawn.

Neurological Scoring

After XMT treatment, the ICH model rats were monitored for appetite, activity and neural defects, and their behavioral disorders were assessed according to the scoring system (Table 1) as mentioned previously (Li et al., 2018).
**Determination of Brain Water Content**

As previously described (Wang et al., 2017), after 72 hours of ICH treatment, the content of brain water was determined by means of dry and wet method. After collecting brain tissues, the samples were divided into five parts: ipsilateral basal ganglia, ipsilateral cortex, contralateral basal ganglia, contralateral cortex and cerebellum. After that, the above samples were immediately weighed to obtain wet weight, and then reweighed for dry weight after drying at 100°C for 24 hours. The percentage of moisture content was calculated by the following formula: \(\frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \times 100\%\).

**TUNEL Staining**

As described previously (Wu et al., 2017), TUNEL and NeuN double staining were used to quantify the apoptotic neurons according to the instructions of the in situ cell death detection kit (Roche, Madison, WI, USA). Three sections of each rat were examined and photographed in parallel for TUNEL-positive cell counting.

**Fluoro-Jade B (FJB) Staining**

The brain slices of SD rats were dewaxed with xylene and a series of graded ethanol solutions. After incubation with 0.06% KMnO4 at room temperature for 15 minutes, the slices were washed with phosphate buffer saline (PBS), dyed with FJB working solution at room temperature for 1 hour, and then dehydrated and air-dried. Finally, the brain slices were observed and photographed under a fluorescence microscope (BX50/BX-FLA/DP70, Olympus, Tokyo, Japan). FJB-positive cells were counted.
Reverse Transcription Quantitative Real-Time PCR (RT-qPCR)

Trizol reagent (15596026, Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA. According to the instructions of the primescript RT Regent Kit (RR047A, Takara Bio Inc., Otsu, Shiga, Japan), the RNA was reverse-transcribed into complementary DNA (cDNA). The synthesized cDNA was detected by RT-qPCR with a Fast SYBR Green PCR kit (Applied Biosystems, Carlsbad, CA, USA) on the ABI PRISM 7300 RT-PCR system (Applied Biosystems, Carlsbad, CA, USA). GAPDH was used as an internal parameter to analyze the relative expression of genes by $2^{ΔΔCt}$ method. The primer design is shown in Table 2.

Hematoxylin-Eosin (HE) Staining

After deep anesthesia, rats were injected rapidly with 0.9% normal saline through the left ventricular aorta until the color of the liver became lighter and the clear fluid flowed out of the right atrial appendage incision. Subsequently, 250 mL of 4% polyoxymethylene (Sigma-Aldrich Chemical Company, St Louis, MO, USA) was used for perfusion until the limbs of rats became stiff. The brain tissues were fixed in 4% paraformaldehyde at 4°C for 24 hours, followed by embedding. The paraffin sections of rat brains were baked in an oven at 60°C for 30 minutes, stained with HE, then sealed with neutral gum, and finally observed under a microscope (Olympus, Tokyo, Japan).

Immunofluorescence

The tissues were cut into 12 μm slices, and the apoptotic neurons were stained with Nissl staining solution. Next, the sections were incubated with the primary antibody cleaved CASP3 (1:100; Cell Signaling Technologies, Beverly, MA, USA) overnight at 4°C. After three washes in PBS, goat anti-rabbit alex594 was applied at room temperature for 1 hour. Afterwards, the sections were
washed with PBS and observed under a Nikon-A1RS confocal microscope.

**Western Blot Assay**

After ICH, the tissues on marginal area of lesions were lysed with the enhanced radioimmunoprecipitation assay (RIPA) lysate containing protease inhibitor (Boster Biological Technology Co., LTD., Wuhan, China), and then the protein concentration was determined with a bicinchoninic acid protein quantitative Kit (Boster Biological Technology Co., LTD., Wuhan, China). The protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the separated protein was transferred to a polyvinylidene fluoride membrane. Following blockade with 5% bovine serum albumin sealed at room temperature for 2 hours to block the non-specific binding, the membrane was incubated overnight at 4°C with diluted rabbit primary antibodies: TNF receptor 1 (TNFR1) (13377S), TNFRSF1A associated via death domain (TRADD) (3694S), TNF receptor associated factor 2 (TRAF2) (4712S) and cleaved-receptor interacting protein 1 (RIP1)/RIP1 (3493S) from Cell Signaling Technologies (Beverly, MA, USA); Fas-associated Death Domain (FADD) (ab24533), caspase-3 (CASP3) (ab4051), cleaved CASP3 (ab49822), extracellular signal-regulated kinase (ERK) (ab17942), phosphorylated (p)-ERK (ab201015), p38 (ab170099), p-p38 (ab47363), c-Jun amino-terminal kinase (JNK) (ab179461), p-JNK (ab124956), and β-actin (ab8227) from Abcam Inc. (Cambridge, MA, USA). The following day, the horseradish peroxidase labeled goat anti-rabbit secondary antibody (ab205719; 1 : 2000; Abcam, Cambridge, MA, USA) was used to incubate the membrane at room temperature for 1 hour. Enhanced chemiluminescence working solution (Millipore, Billerica, Massachusetts, US) was used to visualize protein bands. Image J analysis software was used to quantify the gray level of each band in western blot images with β-actin as internal reference.
Statistical Analysis

Statistical analysis of the data was performed using the statistical software SPSS 21.0 (IBM Corp., Armonk, NY, USA). Measurement data were expressed as mean ± standard deviation. Initially, a test of normality and homogeneity of variance was conducted. Data comparison among multiple groups obeying normal distribution and homogeneous variance was conducted using one-way analysis of variance (ANOVA) or repeated measures of ANOVA, followed by a Tukey’s test. \( p < 0.05 \) demonstrated that the difference was statistically significant.

Results

ICH-Related Target Network

ICH-related target genes were screened from the CTD and GeneCards database and 971 genes at the intersection of two databases were obtained. These 971 overlapping genes were displayed in a PPI network. The network consists of 971 nodes and 34920 edges where each node refers to a gene and each edge connects to two genes (Figure 1). Among them, 20 nodes in red (GAPDH, AKT1, ALB, IL6, INS, VEGFA, TP53, TNF, EGFR, EGF, FN1, MAPK3, MYC, STAT3, CASP3, MAPK8, MAPK1, CXCL8, JUN, MMP9) had high degree with more edges: 501 for GAPDH, 489 for AKT1, 488 for ALB, 471 for IL6, 462 for INS, 433 for VEGFA, 426 for TP53, 412 for TNF, 389 for EGFR, 375 for EGF, 367 for FN1, 361 for MAPK3, 337 for Myc, 337 for STAT3, 329 for CASP3, 309 for MAPK8, 308 for MAPK1, 307 for CXCL8, 305 for JUN and 294 for MMP9. These indicated that these 20 genes are at the core of gene network and thus they may be the key genes in the cause and development of ICH.

TCM-Compound-Target Network
To further screen TCM related branches, we performed analysis on TCM-compound-target and established another network. The TCM-compound-target network consists of 261 nodes and 4139 edges (Figure 2), including 7 kinds of TCMs, 65 compounds and 189 targets. In this network, we found that many targets are regulated by various compounds at the same time, such as PTGS2, AKT1, TP53, ESR1, CASP3, Jun, and TNF, but there are also targets, such as IL-6, ECE1, CLDN4, CTRB1, NPEPPS, CACNA2D1, PCOLCE, ACPP, EIF6, PKIA, only regulated by one compound. For example, PTGS2 was regulated by all compounds in TCM except Euscaphis japonica and Alisma Orientale (Sam.) Juz, while IL-6 was only regulated by quercetin in Gardeniae Fructus. These results suggest that the compounds in XMT may act synergistically on these targets, thereby exerting a pharmacological effect on the treatment of ICH. In addition, it also suggests the multi-component, multi-target and multi-disease therapeutic characteristics of TCM.

Cluster Analysis for TCM-Compound-Target Network

TCM-compound-target network was next assessed by cluster analysis. We used Cytoscape plug-in MCODE, we clustered the relation on the TCM-compound-target network for analysis and obtained eight topological modules (Figure 3): Cluster 1: score = 43.36, Nodes = 51, Edges = 1085; Cluster 2: score = 6.933, Nodes = 16, Edges = 52; Cluster 3: score = 5.474, Nodes = 20, Edges = 52; Cluster 4: score = 5.2, Nodes = 6, Edges = 13; Cluster 5: score = 5.077, Nodes = 14, Edges = 33; Cluster 7: score = 3, Nodes = 5, Edges = 6; Cluster 8: score = 3, Nodes = 3, Edges = 3.

Key Target-Compound-TCM-XMT Network

Subsequently, we retrieved XMT potential targets from ICH-related target network and TCM-compound-target network where the genes at the intersections were selected. A total of 121 potential targets for XMT treatment of ICH were obtained (Figure 4A). Further screening
conditions for these targets were set at BC $\geq$ Avg (BC), CC $\geq$ Avg (CC) and De $\geq$ Avg (DE). As a result, we obtained 34 key targets (MAPK8, JUN, TP53, CASP3, TNF, EGFR, EGF, Fos, SERPINE1, EDN1, AKT1, CCND1, VEGFA, STAT3, RELA, IL1B, IL6, IL10, MYC, MAPK1, ERBB2, IL4, CCL2, ESR1, MMP9, ICAM1, STAT1, AR, NR3C1, PPARG, PTGS2, MMP2, CAT, MPO) (Figure 4B). These 34 key targets correspond to 55 compounds and 7 TCM in XMT (Figure 4C).

**KEGG Enrichment Analysis and Key Signal Pathway Target-Compound-Drug Network**

Through DAVID database, the 121 candidates genes for XMT treatment of ICH obtained above were analyzed by KEGG analysis (Figure 5A). XMT mainly affected 91 pathways including: (hsa05200) pathways in cancer, (hsa04668) TNF signaling pathway, (hsa05161) hepatitis B, (hsa05212) pancreatic cancer, (hsa05219) bladder cancer, (hsa05142) Chagas disease (American trypanosomias), (hsa05205) proteoglycans in cancer, (hsa04066) HIF-1 signaling pathway, (hsa05140) leishmaniasis, (hsa05133) Pertussis, (hsa05210) colorectal cancer, (hsa05321) inflammatory bowel disease, (hsa04010) MAPK signaling pathway, (hsa04917) prolactin signaling pathway, (hsa04620) Toll-like receptor signaling pathway, (hsa05213) endometrial cancer, (hsa05164) influenza A, (hsa05215) prostate cancer, (hsa04380) osteoplast differentiation, (hsa05160) and hepatitis C ($p < 0.05$). TNF signaling pathway is the main signaling pathway in the treatment of ICH with XMT. There are 14 key targets enriched in TNF signaling pathway. These 14 key targets correspond to 49 compounds and 5 TCM in XMT (Figure 5B).

**XMT Regulated the TNF Signaling Pathway in the Cerebral Ischemic Area upon ICH**

We then explored the potential regulatory role of XMT on the TNF signaling pathway in cerebral ischemic area. As illustrated in Figure 6A, regarding the TNF signaling pathway, TNFR1
was indicated to regulate the expression of CASP3 through death inducing signaling complex (DISC/complex 2), including TRADD, TRAF2, FADD and RIP1. Meanwhile, TNFR1 regulated the expression of CASP3 through signalosome (complex 1) including TRADD, TRAF2 and RIP1, which regulated MAPK and NF-κB signaling pathways and their downstream genes (CCL2, IL1B, IL6, TNF, Fos, Jun, MMP9, EDN1, ICAM1, PTGS2). Figure 6B showed the ischemic core area and ischemic area at the site of cerebral hemorrhage. The neurological scoring (Figure 6C) showed that compared with sham-operated rats, the ICH rats had notably impaired neurobehavioral ability \( (p < 0.05) \) while ICH rats could partially alleviate the injury and restore neurobehavioral ability under the XMT treatment at a dose of 42 mg/kg or 84 mg/kg \( (p < 0.05) \). The effect of XMT was similar to that of Edaravone. Compared to sham-operated rats, western blot assay revealed that the protein expression of TNFR1, TRADD, TRAF2, FADD and RIP1 and the extent of RIP1 phosphorylation were significantly increased in ICH rats \( (p < 0.05) \), while the XMT group was obviously decreased \( (p < 0.05; \text{Figure 6D, E}) \). Meanwhile, the results of immunofluorescence assay for TNFR1 were consistent with those of Western blot assay. XMT markedly inhibited the abnormal increase of TNFR1 protein expression in cerebral ischemic area induced by ICH \( (p < 0.05; \text{Figure 6F and supplementary Figure 1}) \). Collectively, XMT significantly alleviated aberrant expression of TNFR1 following ICH and cerebral ischemia.

XMT Inhibited the Apoptosis of Neurons in Ischemic Area by Regulating TNFR1/CASP3

In addition, we investigated the effect of XMT on TNFR1/CASP3 in ischemic area through network pharmacology. It was shown that XMT mainly regulated the expression of DISC and its downstream CASP3 through the TNF signaling pathway to inhibit the apoptosis of neurons (Figure 7A). To verify the mechanism revealed by network, we established rat model and administered rats with XMT to observe alteration of CASP3. Compared with that in sham-operated rats, ICH rats exhibited elevated expression of cleaved CASP3/CASP3 in ICH rats \( (p < 0.05) \), while
administration of 42 mg/kg or 84 mg/kg of XMT notably decreased cleaved Cleaved CASP3/CASP3 level ($p < 0.05$; Figure 7B). At the same time, the results of immunofluorescence assay for cleaved CASP3 were consistent with Western blot assay revealing increased Cleaved CASP3/CASP3 level in the ICH rats. Upon administration with XMT, the abnormal increase of cleaved CASP3 protein expression in the ischemic area secondary to ICH was decreased ($p < 0.05$; Figure 7C and supplementary Figure 2). These results suggest that XMT may inhibit the apoptosis of neurons by regulating the protein expression of cleaved CASP3.

**XMT Inhibited the Degeneration and Apoptosis of Neurons in Ischemic Area by Regulating Cleaved CASP3**

To explore how XMT regulates neurons progression upon ICH through cleaved CASP3, we stained the neurons with FJB and TUNEL double staining to further observe the effect of XMT on the degeneration and apoptosis. The results revealed that compared with sham-operated rats, the number of TUNEL positive cells ($p < 0.05$; Figure 8A) and FJB-positive cells ($p < 0.05$; Figure 8B) was significantly increased in the ICH rats, while ICH rats treated with XMT or Edaravone had decreased TUNEL- and FJB-positive cells (supplementary Figure 3). The effect of 84 mg/kg XMT was similar to 2 mg/kg Edaravone, but 84 mg/kg XMT was more effective. The above experimental results demonstrated that XMT has a certain rescue effect on the lesion of ICH, more effective than Edaravone if at the same dose. Of note, XMT inhibited apoptosis and degradation of ischemic nerve cells.

**XMT Inhibited the TNFR1/NF-κB Pathway upon ICH**

Next, we analyzed the regulatory effect of XMT on TNFR1/MAPK pathway in ischemic area. Network pharmacology revealed that XMT can regulate the abnormal activation of NF-κB signaling
pathway mediated by signalosome through TNF signaling pathway (Figure 9A). We therefore determined the expression of related proteins and downstream genes in NF-κB signaling pathway. Western blot assay showed that compared with sham-operated rats, ICH rats exhibited increased protein expression of p-p65/p65 and p-IκBα/IκBα ($p < 0.05$). In addition, treatment with XMT had notably decreased protein expression of p-p65/p65 and p-IκBα/IκBα ($p < 0.05$; Figure 9B). Moreover, ICH rats also displayed increased expression of inflammatory cytokines; IL6, IL1B, TNF (Figure 9C), adhesion molecule ICAM1 (Figure 9D), and PTGS2 (Figure 9E) in comparison to that of sham-operated rats ($p < 0.05$), while the expression of all these genes was notably decreased in ICH rats treated with XMT relative to that in ICH rats ($p < 0.05$). The aforementioned results validated our network pharmacology results that XMT could block the TNFR1/NF-κB pathway in ischemic area.

**XMT Suppressed the TNFR1/MAPK Pathway in Ischemic Area**

Next, we analyzed the interaction between XMT and TNFR1/MAPK pathway in ischemic area. The network pharmacology showed that XMT alleviated the abnormal activation of signalosome-mediated MAPK signaling pathway through TNF signaling pathway (Figure 10A). In order to verify the result of network, the expression of related proteins and downstream genes in MAPK signaling pathway in rats was determined by Western blot assay. p-ERK/ERK, p-p38/p38 and p-JNK/JNK was highly expressed in ICH rats relative to sham-operated rats ($p < 0.05$). Addition of XMT reduced protein expression of p-ERK/ERK, p-p38/p38 and p-JNK/JNK ($p < 0.05$; Figure 10B). Compared with that in sham-operated rats, the expression of Fos and Jun ($p < 0.05$; Figure 10C), CCL2 ($p < 0.05$; Figure 10D), MMP9 ($p < 0.05$; Figure 10E) and EDN1 in ICH rats was significantly higher ($p < 0.05$; Figure 10F), while XMT administration decreased the expression of the above factors as well ($p < 0.05$). All these results verified our network pharmacology results that XMT could inhibit the TNFR1/MAPK pathway in ischemic area.
XMT Alleviated Inflammation, Edema and Increased Perfusion in Cerebral Ischemic Area

Pro-inflammatory factors, chemokines and MMPs, are all related to the destruction of BBB (Lasek-Bal et al., 2019; Zhang et al., 2018). At the same time, the destruction of BBB can further induce inflammation by promoting leukocyte infiltration. In addition to promoting inflammation, the destruction of BBB can also lead to the formation of angiogenic edema after ICH. Whether XMT alleviates the formation of angiogenic edema following ICH by inhibiting inflammation and MMPs remained elusive and aroused our interest. We applied HE staining and tissue water content measurement to assess the effect of XMT. In sham-operated rat, brain tissues contained rich neurons and the neurons were arranged orderly with normal tissue gaps while the staining of cell tissue structure was uniform with clear nucleus (blue). In ICH rats and ICH rats treated with vehicle, glial cells and neurons were swollen, decreased and unevenly arranged, along with widened extracellular space, and weakened eosinophilic of cytoplasm; some cells were pyknotic and necrotic and the staining was deepened, accompanied by infiltration of a large number of neutrophils and microglial cells. Upon treatment with XMT or Edaravone, all symptoms were alleviated: the swelling was attenuated, the widening shrunk, the eosinophilic of cytoplasm was enhanced, and the number of pyknosis necrosis cells and inflammatory cells was reduced (Figure 11A). Meanwhile, the water content of brain tissues in the ischemic area was notably higher in ICH rats than in sham-operated rats ($p < 0.05$). Relative to the ICH rats, rats treated with XMT or Edaravone displayed a decline in the water content of brain tissues in the ischemic area ($p < 0.05$; Figure 11B).

Discussion

ICH is a fatal neurological disorder with poor prognosis (Kim et al., 2014). In spite of modern treatment effectively realizing rapid stabilization, management of patients with ICH requires
long-term anticoagulation and patients are prone to other neurodegenerative diseases. Novel medical and surgical approaches are urgently required (Schrag and Kirchner, 2007). Notably, network pharmacology in combination with TCM research has been highlighted as novel insights in the development of modern drug (Wu and Wu, 2015). In this study, we elucidated that XMT ameliorates the secondary damage post ICH through modulation of TNFR1/CASP3, TNFR1/NF-κB and TNFR1/MAPK.

Our study identified the TNF signaling pathway as the main signal pathway in the treatment of ICH with XMT. We also predicted 14 TNF signaling pathway-enriched genes (ICAM1, IL6, TNF, CCL2, PTGS2, RELA, MMP9, EDN1, MAPK1, Fos, CASP3, JUN, IL1B, MAPK8) as key targets for the intervention efficacy on ICH. Furthermore, we found that XMT-mediated TNFR1/CASP3, TNFR1/NF-κB and TNFR1/MAPK were involved in the neuron apoptosis, inflammation/edema and in ischemic area post ICH. TNFR1, one of the TNF signaling receptors, is accountable for the pathogenesis of Alzheimer's disease through facilitating neuron death (Steeland et al., 2018). CASP3 is a well-known pro-apoptotic gene (Yamabe et al., 1999), and its activation has been implicated in ischemic stroke (Li et al., 2020). Moreover, upregulated plasma level of TNFR1 was correlated with incident ICH, suggesting the underlying association between TNF-mediated inflammation and vascular changes prior to ICH (Svensson et al., 2017). Consistently, downregulated expression of TNF-α, TNFR1, and NF-κB by cannabidiol in the total, core, and ischemic areas could play a cerebroprotective role in ischemic injury (Khaksar and Bigdeli, 2017). Additionally, inactivation of the AMPK/JNK/p38 MAPK pathway by melanocortin receptor 4 with RO27-3225 was found to reduce neuroinflammation following ICH in a mouse model (Chen et al., 2018). The inactivated TLR4/TRA6/NF-κB pathway by Luteolin also ameliorates neuroinflammation post ICH (Yang et al., 2020). Furthermore, downregulation of TNF-α and TNFR1 was found to attenuate endothelial necroptosis and improve stroke outcomes (Chen et al., 2019). EDN1 is well-known for its vasoconstrictor function in controlling brain microcirculation.
(Petrov et al., 2002). Interference with EDN1 could also improve cerebral hypoperfusion and restore CBF in multiple sclerosis (D'Haeseleer et al., 2013). Overall, these previous reports are supportive of our finding regarding the involvement of TNFR1/CASP3, TNFR1/NF-κB and TNFR1/MAPK in ICH.

Mechanistically, our study unfolded that XMT could mediate the TNF signaling pathway, mainly by regulating TNFR1/CASP3, TNFR1/NF-κB and TNFR1/MAPK in the ischemic area post ICH. In fact, another TCM prescription, Danggui-Honghua, was also detected through network analysis. It was considered as a promising therapy for blood stasis syndrome by targeting genes mainly enriched in the TNF signaling pathway (Yue et al., 2017). Additionally, Longxuetongluo capsule, a prescription for ischemic stroke treatment, could attenuate neuro-inflammation in BV2 microglia cells partially by diminishing IL-1β, IL-6 and TNF-α production and NF-κB translocation (Hong et al., 2020). The regulatory of XMT on TNF signaling pathway-related genes has been reported but not extensively elucidated. Zhang et al. found that XMT granules could diminish IL-6, TNF-α, and hsCRP expression, which ameliorated carotid atherosclerosis (Zhang et al., 2014). A previous study has found that Xuemaitong granules could decrease the expression of MMP-1, MMP-9 and endothelin in a rabbit model of atherosclerosis, thereby improve blood rheology (Qi et al., 2005). In addition, many of the compounds in XMT have been revealed to involve in the regulation of TNF signaling pathway-related genes. For instance, AA-24-a extracted from A. Orientale (Sam.) Juz. was found to be capable of elevating glucose uptake via the CaMKKβ-AMPK-p38 MAPK/AS160 pathway (Chen et al., 2020). Similarly, Radix Puerariae-derived puerarin conferred renal protection against cisplatin nephrotoxicity by inactivating the TLR4/NF-κB pathway (Ma et al., 2017). Collectively, it was concluded that XMT yields therapeutic efficacy on ICH via regulation of TNFR1/CASP3, TNFR1/NF-κB and TNFR1/MAPK.

In conclusion, the results obtained from this study demonstrated that XMT is able to alleviate
secondary damage in the ischemic area post ICH through mediating TNFR1/CASP3, TNFR1/NF-κB and TNFR1/MAPK. This finding also suggests the role of network pharmacology in analyzing the molecular mechanism of TCM in treatment of stroke. However, the clinical efficacy of the therapeutic targets still needs further validation in clinical research.
References


Financial Disclosure

No author has an actual or perceived conflict of interest with the contents of this article.

Funding

This work was supported by the Science and Technology Foundation of Guizhou Province (No. [2017]1015), and the Project of Science and Technology of Guizhou Educational Committee (No. KY[2018]053).

Ethics Statement

The present study was approved by the Ethics Committee of the Animal of Guizhou Medical University. All animal experiments were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Acknowledgments

We acknowledge and appreciate our colleagues for their valuable suggestions and technical assistance for this study.

Conflicts of Interests

The authors declare that they have no conflicts of interests.
Author’ Contribution

Participated in research design: Zhang

Conducted experiments: Zeng.

Contributed new reagents or analytic tools: Zhang, Zeng, and Wu.

Performed data analysis: Wu

Wrote or contributed to the writing of the manuscript: Zhang, Zeng, and Wu.

Consent for Publication

Not applicable.

Availability of Data and Materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Details

1 Neurosurgery Department, Huiya Hospital of the First Affiliated Hospital, Sun Yat-Sen University, Huizhou 516081, P. R. China

2 Neurosurgery Department, the First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine, Guiyang 550006, P. R. China

3 Graduate School, Guizhou University of Traditional Chinese Medicine, Guiyang 550025, P.
R. China
Legends

**Figure 1** Network of ICH-related targets. Each circle in the figure represents a gene, and each edge connects to two genes that bind to each other. The increasing number of edge responses to higher degree. Core genes (red) have higher degrees.

**Figure 2** TCM-compound-target network. This network contains 7 kinds of TCMs, 65 compounds, and 189 targets (The red V-shaped nodes represent TCM, the pink hexagons represent compounds, and the purple circles represent targets).

**Figure 3** Cluster analysis for TCM-compound-target network. The pink hexagons represent compounds, and the purple circles represent targets.

**Figure 4** Key target-compound-TCM-XMT network. A, 121 putative targets for XMT treatment of ICH. B, 34 key targets for XMT treatment of ICH. C, Key target-compound-TCM-XMT network (The red V-shaped nodes represent TCM, the pink hexagons represent compounds, and the purple circles represent targets).

**Figure 5** KEGG enrichment analysis through DAVID and key signal pathway target-compound-drug network. A, KEGG enrichment analysis. B, Key signal pathway target-compound-drug network. The red V-shaped nodes represent TCM, the pink hexagons represent compounds, and the purple circles represent targets.

**Figure 6** XMT inhibited TNFR1 aberrant expression and cerebral ischemia upon ICH. A, TNF signaling pathway. B, Ischemic area and core area. C, Neurological function scoring. D & E, Western blot assay for detection of the protein expression of TNFR1, TRADD, TRAF2, FADD and RIP1 and the extent of RIP1 phosphorylation in ischemic area. F, Quantification of the protein expression and location of TNFR1 in ischemic area. * $p < 0.05$ vs. sham-operated rats. These data were measurement data, expressed as mean ± standard deviation. Data comparison among multiple groups were conducted using one-way ANOVA, followed by a Tukey’s test. All experiments were repeated three times.
**Figure 7** XMT decreased the apoptosis of neurons in ischemic area by regulating the TNFR1/CASP3 signaling pathway. A, Diagrammatic sketch of the TNFR1/CASP3 signaling pathway. B, Western blot analysis of Cleaved caspase-3 and caspase-3 in the ischemic area. C, Quantification of Cleaved caspase-3 in the ischemic area. *p < 0.05 vs. sham-operated rats. #p < 0.05 vs. ICH rats. These data were measurement data, expressed as mean ± standard deviation. Data comparison among multiple groups were conducted using one-way ANOVA, followed by a Tukey’s test. All experiments were repeated three times.

**Figure 8** XMT inhibited the degeneration and apoptosis of neurons in cerebral ischemic area by regulating cleaved CASP3. A, Quantification of the degeneration of neurons in the ischemic area. B, Quantification of the apoptosis of neurons in the ischemic area. *p < 0.05 vs. sham-operated rats. #p < 0.05 vs. ICH rats. These data were measurement data, expressed as mean ± standard deviation. Data comparison among multiple groups were conducted using one-way ANOVA, followed by a Tukey’s test. All experiments were repeated three times.

**Figure 9** XMT suppressed the TNFR1/NF-κB pathway in cerebral ischemic area. A, Schematic diagram of TNFR1/NF-κB signaling pathway. B, The protein expression of p65 and IκBα as well as the extent of p65 and IκBα phosphorylation in ischemic area as detected by Western blot assay. C-E, The mRNA expression of IL6, IL1B, TNF, ICAM1 and PTGS2 as detected by RT-qPCR. *p < 0.05 vs. sham-operated rats. #p < 0.05 vs. ICH rats. These data were measurement data, expressed as mean ± standard deviation. Data comparison among multiple groups were conducted using one-way ANOVA, followed by a Tukey’s test. All experiments were repeated three times.

**Figure 10** XMT blocked the TNFR1/MAPK pathway in cerebral ischemic area. A, Schematic diagram of TNFR1/MAPK signaling pathway. B, The protein expression of ERK, p38 and JNK as well as the extent of ERK, p38 and JNK phosphorylation in ischemic area as detected by Western blot assay. C-F, The mRNA expression of Fos and Jun, CCL2, MMP9 and EDN1 as detected by RT-qPCR. *p < 0.05 vs. sham-operated rats. #p < 0.05 vs. ICH rats. These data were measurement data, expressed as mean ± standard deviation. Data comparison among multiple groups were conducted using one-way ANOVA, followed by a Tukey’s test. All experiments were repeated three times.
data, expressed as mean ± standard deviation. Data comparison among multiple groups were conducted using one-way ANOVA, followed by a Tukey’s test. All experiments were repeated three times.

**Figure 11** XMT alleviated inflammation, edema and increases perfusion in cerebral ischemic area. A, HE staining for the cerebral ischemic area. B, Water content measurement in ipsilateral basal ganglia, ipsilateral cortex, contralateral basal ganglia, contralateral cortex and cerebellum. *p < 0.05 vs. sham-operated rats. #p < 0.05 vs. ICH rats. These data were measurement data, expressed as mean ± standard deviation. Data comparison among multiple groups were conducted using one-way ANOVA, followed by a Tukey’s test. All experiments were repeated three times.
### Table 1 Behavior and activity scores

<table>
<thead>
<tr>
<th>Category</th>
<th>Behavior</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appetite</td>
<td>Finished meal</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Left meal unfinished</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Scarcely ate</td>
<td>2</td>
</tr>
<tr>
<td>Activity</td>
<td>Walk and reach at least three corners of the cage</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Walk with some stimulations</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Almost always lying down</td>
<td>2</td>
</tr>
<tr>
<td>Deficits</td>
<td>No deficits</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Unstable walk</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Impossible to walk</td>
<td>2</td>
</tr>
<tr>
<td>Gene name</td>
<td>Primer sequences (5′-3′)</td>
<td>Gene name</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IL-6</td>
<td>sense CCAAGAGGTGAGTGCTTCCC</td>
<td>TNF-α</td>
</tr>
<tr>
<td></td>
<td>antisense CTGTTGTCAGACTCTCTCCCT</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>sense TCGCACTGATCACAACAAGAG</td>
<td>ICAM1</td>
</tr>
<tr>
<td></td>
<td>antisense TGCTCATGTCCTCCTGTCGAAG</td>
<td></td>
</tr>
<tr>
<td>PTGS2</td>
<td>sense CTGGTGCTTGTCTGTGATGTATGATG</td>
<td>FOS</td>
</tr>
<tr>
<td></td>
<td>antisense GGATGCTCCTGTTGATGTATGTCG</td>
<td></td>
</tr>
<tr>
<td>JUN</td>
<td>antisense CAGATTCAAAGGTGGAAGAG</td>
<td>CCL2</td>
</tr>
<tr>
<td></td>
<td>sense CCAAGAGGCTGTAGTTATTTG</td>
<td>MMP9</td>
</tr>
<tr>
<td></td>
<td>antisense TCTGGAAAGAGCTTGTTT</td>
<td>EDN1</td>
</tr>
<tr>
<td></td>
<td>sense CTGGTGACTTCCGACCTG</td>
<td>β-Actin</td>
</tr>
<tr>
<td></td>
<td>antisense AACAGTCCGCCCTAGAAGCA</td>
<td></td>
</tr>
</tbody>
</table>

Note: IL-6, interleukin 6; TNF-α, tumour necrosis factor-α; IL-1β, interleukin 1β; ICAM1, intercellular adhesion molecule 1; PTGS2, prostaglandin-endoperoxide synthase 2; JUN, jun proto-oncogene; CCL2, C-C motif chemokine ligand 2; MMP9, Matrix metalloproteinase 2; EDN1, endothelin-1.
Figure 5

A

B

Pathways in cancer
TNF signaling pathway
Hepatitis B
Pancreatic cancer
Bladder cancer
Chagas disease (American trypanosomiasis)
Proteoglycans in cancer
HIF-1 signaling pathway
Leishmaniasis
Pertussis
Colorectal cancer
Inflammarory bowel disease (IBD)
MAPK signaling pathway
Prolactin signaling pathway
Toll-like receptor signaling pathway
Endometrial cancer
Influenza A
Prostate cancer
Osteoclast differentiation
Hepatitis C

Figure 5

This article has not been copyedited and formatted. The final version may differ from this version.

JPET Fast Forward. Published on December 11, 2020 as DOI: 10.1124/jpet.120.000083

Downloaded from jpet.aspetjournals.org at ASPET Journals on July 6, 2021
Figure 6

A

B

C

D

E

F

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 7

**A**

**B**

**C**

Relative protein expression of cleaved-caspase3/Caspase-3

Cleaved caspase-3 positive cells (cells/field)

* * *

# # #

This article has not been copyedited and formatted. The final version may differ from this version.

JPET Fast Forward. Published on December 11, 2020 as DOI: 10.1124/jpet.120.000083
Figure 8

A

B

Percentage of apoptotic neurons (%)

F: Nnumbers/mm²

Sham ICH ICH + Vehicle ICH + XMT21 ICH + XMT42 ICH + XMT84 ICH + Edara

Sham ICH ICH + Vehicle ICH + XMT21 ICH + XMT42 ICH + XMT84 ICH + Edara

* # # #
Figure 11

A

H&E

Sham 21 42 84 2

B

Brain water content (%)
A Network Pharmacology-Based Analysis of the Protective Mechanism of Miao Medicine Xuemaitong Capsule against Secondary Brain Damage in the Ischemic area Surrounding Intracerebral Hemorrhage

Running title: Protective mechanism of Xuemaitong Capsule against ICH

Bo Zhang 1,2,#,* , Zhengyan Zeng 3,#, Haijun Wu 2,#

1 Neurosurgery Department, Huiya Hospital of the First Affiliated Hospital, Sun Yat-Sen University, Huizhou 516081, P. R. China

2 Neurosurgery Department, the First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine, Guiyang 550006, P. R. China

3 Graduate School, Guizhou University of Traditional Chinese Medicine, Guiyang 550025, P. R. China

# Bo Zhang & Zhengyan Zeng & Haijun Wu are co-first authors

* Correspondence to: Bo Zhang Neurosurgery Department, Huiya Hospital of the First Affiliated Hospital, Sun Yat-Sen University, No. 186, Northern Zhongxing Road, Dayawan District, Huizhou 516081, Guangdong Province, P. R. China; Neurosurgery Department, the First Affiliated Hospital of Guizhou University of Traditional Chinese Medicine, No. 71, Baoshan North Road, Yunyan District, Guiyang 550006, Guizhou Province, P. R. China

E-mail: zhbbls@163.com
Tel: +86-0752-6516990-8160
FIGURE S3

A

Sham  ICH  ICH + Vehicle  ICH + XMT21  ICH + XMT42  ICH + XMT84  ICH + Edara

B

Sham  ICH  ICH + Vehicle  ICH + XMT21  ICH + XMT42  ICH + XMT84  ICH + Edara