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Nrf2 regulates microglia-mediated phagocytosis and neuroinflammation after intracerebral hemorrhage

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Abstract: Activated microglia is essential for hematoma clearance and recovery after intracerebral hemorrhage (ICH). This study aims to evaluate the effect of microglial functional transformation in hematoma clearance after ICH. Through in vitro and in vivo experiments, we also investigate whether Nuclear factor erythroid 2-related factor 2 (Nrf2) -mediated microglial phagocytosis and inflammatory response plays a role in hematoma clearance and functional recovery after ICH. In vitro experiments, BV-2 cells were cultured and randomly divided into 4 groups, including normal control, microglia + Nrf2-siRNA (100 nmol/L), microglia + monascin (15 µM), and microglia + Xuezhikang (200 µg/mL) groups. In vivo experiments, 42 mice were divided into 2 groups, i.e., sham, ICH+vehicle, ICH+Nrf2-/-, ICH+monascin (10mg/kg/day, twice) and ICH+Xuezhikang (0.2g/kg/day, twice) groups. Further, neurologic scores, hemoglobin levels, microglial phagocytosis, brain expression of CD80/Trem1/TNF-α (pro-inflammatory cytokines), and CD206/Trem2/BDNF (anti-inflammatory cytokines) were analyzed 72 hours after surgery. The results showed that Nrf2 agonists improved neurological deficits and decreased hemoglobin levels after ICH through regulating microglial functional transformation. Administration of Nrf2 agonist-monascin/ Xuezhikang improved microglia-mediated phagocytosis of erythrocytes and bio-particles through Nrf2 upregulation. Alternatively, monascin/ Xuezhikang promoted the expression of Triggering receptor II expressed on myeloid cells (Trem2), CD206, and BDNF while inhibiting the expression of Trem1,CD80, and TNF-α in microglia. Conversely, Nrf2 inhibition (Nrf2 siRNA or Nrf2-/-) demonstrated conflicting results after ICH. Microglial functional transformations are implicated in hematoma clearance after ICH. Nrf2 activation leads to microglial functional transformation and phagocytic responses then exert its neuroprotection after ICH. Nrf2 activator (Monascin /Xuezhikang) improves hematoma clearance and alleviates neuroinflammation by regulating microglial functional alteration after ICH.

Keywords: Microglia; Phagocytosis; Neuroinflammation; Intracerebral hemorrhage; Trem1; Trem2.

1. Introduction

Intracerebral hemorrhage (ICH) is a devastating stroke subtype, with high morbidity and mortality [1-3]. Accumulating evidence suggests that hematoma and its degradation products primarily cause a poor prognosis after ICH[4]. Recent studies show that microglia and endogenous scavenging system [5], (a novel effective therapeutic approach for ICH) regulate the process of hematoma clearance[4,6].
Microglia are activated by diverse pathologic events or changes in brain homeostasis. Activated microglia are divided into pro-inflammatory and anti-inflammatory functional states after ICH. Pro-inflammatory phenotype is characterized by the production of tumor necrosis factor-α (TNFα), interleukin 1β (IL-1β), CD80, CD86, and CD16/32, etc.; it induces neuron cell death and promotes neuroinflammation. On the other hand, anti-inflammatory phenotype attenuates inflammation, manipulates phagocytosis, and promotes neural repair in the presence of IL-4, IL-10, brain-derived neurotrophic factor (BDNF), and CD206, etc. [7-9]. Triggering receptor I expressed on myeloid cells (Trem1) is a crucial inflammatory amplifier of microglia [10,11]. On the other hand, triggering receptor II expressed on myeloid cells (Trem2) exerts anti-inflammatory and neuroprotective effects. It might be a receptor that regulates phagocytosis [12,13].

Nuclear factor erythroid 2-related factor 2 (Nrf2) and peroxisome proliferator-activated receptor γ (PPARγ) has attracted significant research attention due to its role in the clearance of endogenous hematomas as regulators [14,15]. Besides, they potentially promote the removal of erythrocytes and hematoma remnants by interacting with Trem1 or Trem2.

In our previous work, we confirmed that PPARγ-Nrf2 agonist-monascin facilitates hematoma clearance and exerts neuroprotection after ICH [16,17]. Nonetheless, studies on the underlying mechanism of hematoma clearance and its neuroprotection of Nrf2 have not reached maturity. Therefore, this research further evaluated the natural Nrf2 expression as well as its relationship with Trem2 and Trem1 after ICH. Then, we explored the mechanism and effects of Nrf2 on microglial phagocytosis and neuroinflammation using Nrf2-siRNA in vitro and Nrf2 Knockout mice (Nrf2-/-) in vivo or Nrf2 agonists monascin/Xuezhikang. Notably, monascin here acts as a novel dual agonists of Nrf2 and PPAR-γ whereas Xuezhikang is an extract of monascin.

2. Materials and Methods
2.1a. Cellular materials
Experimental cells: BV-2 cells (Solebo)

2.2a. Cellular experimental methods
2.2.1. Experimental cell groups: Normal group, siRNA group, monascin group, Xuezhikang group
2.2.2. Cell culture
BV-2 cells were cultured in a humidified incubator with 5% CO2 at 37 °C. After 2-3 days, Dulbecco’s Modified Eagle Medium (DMEM) was replaced, and non-adherent cells were removed. When the primary cultures reached 80% confluence, cells were harvested using 0.25% trypsin–EDTA solution and sub-cultured. Thereafter, the third passage of cells was selected for subsequent experiments.

2.2.3. Screening of Monascin/Xuezhikang concentration and the transfection sequence of Nrf2-siRNA by qPCR
RT-PCR was used to detect the Nrf2-mRNA levels, screen its best sequence, and the best drug concentrations of monascin or Xuezhikang. Exactly 1 ml aliquots of 5, 15, 30 μmol/L monascin or 100, 200, 500μg/ml Xuezhikang was added to a 12-well plate with three multiple holes in each group, and Nrf2 interference vector(siNrf2) were synthesized (GenePharma, Shanghai,
China), then transfected by incubating in DMEM containing GP-transfect-Mate (GenePharma, Shanghai, China).

Total RNA was extracted using the Trizol reagent. First-strand cDNA was synthesized using the PrimeScript RT Master Mix Kit (ABI-Invitrogen, Thermo Fisher Scientific, Grand Island, NY, USA), while β-Actin was used as the internal control. The quantification of endogenous control mRNA levels was performed using TaqMan assays. The data were analyzed using the \( 2^{-\Delta\Delta Ct} \) method.

The Nrf2-siRNA (100 nM) primer sequences screened by RT-PCR exhibited sense chain GCAGGACAUGGAUUUGAUUTT and antisense chain AAUCAAAUCCAUGUCCUGCTG. Then, 15μmol/L monascin or 200ug/ml Xuezhikang were selected for subsequent experiments.

2.2.4. Cell immunofluorescence

The immunofluorescence was performed as previously described[38]. The BV2 microglial cells were treated using Nrf2-siRNA, monascin, and Xuezhikang, respectively. The cells were collected and fixed with 4% paraformaldehyde, permeabilized using 0.5% Triton X-100, then blocked with goat serum for 60 min. The samples were overnight incubated with primary antibodies (anti-Nrf2, anti-Trem1, anti-Trem2, anti-CD80, anti-CD206, Abcam) at 4 °C, incubated with a fluorescent dye-conjugated secondary antibody in the dark for 1 h, then stained using 4’,6-diamidino-2-phenylindole (DAPI). Three different visual fields were randomly selected for each sample and photographed under a fluorescence microscope (LeicaDmill, Germany).

2.2.5. Phenotype of BV-2 cells detected by flow cytometry

The BV-2 cells were inoculated and 100 µL cell suspension with a final concentration of 10 × 10⁶ cells/mL was pipetted into a round-bottom Eppendorf tube. After incubation with the primary antibody CD80/CD206 (anti-CD80 1:50, anti-CD206 1:50 Abcam) for 2h, the microglial cells were washed then incubated using a fluorescent-labeled secondary antibody for 1h. The total percentage of CD206 and the relative ratio of CD206 to CD80 were determined through flow cytometry. Notably, CD206 was shown as APC in the Q1 quadrant; CD80 was displayed as fluorescein isothiocyanate (FITC) in the Q4 quadrant. The common mark was in the Q2 quadrant. The percentage of CD206 and CD80 (%) = 100 × (Q1+Q2)/(Q2+Q4) was the phenotype of the microglia. The phenotype of the cells was evaluated by quantitative percentage.

2.2.6. Detection of Nrf2, Trem1, Trem2, CD80, CD206, TNF-α, and BDNF protein expressions via Western Blot (WB)

The BV2 cells were collected and treated with radioimmunoprecipitation (RIPA) lysis buffer and measured using a bicinchoninic acid (BCA) kit as previously described[39]. Equal amounts of protein were loaded on an SDS polyacrylamide gel, electrophoresed, then transferred to polyvinylidene fluoride (PVDF) membranes, which were then overnight incubated with primary antibodies (anti-Nrf2, anti-Trem1, anti-Trem2, anti-CD80, anti-CD206, anti-TNF-α, and anti-BDNF, Abcam) at 4 °C. The membranes were incubated with a horseradish peroxidase-conjugated secondary antibody and visualized through chemiluminescence. Densitometry was performed to quantify the signal intensity using ImageJ software (https://imagej.net).

2.2.7. Observation of phagocytosis of fluorescent bioparticles by immunofluorescence

The BV2 cells were treated with 10 mg/mL zymosan fluorescent bioparticles (Alexa Fluor594 conjugate; zymosan: BV-2 = 40:1) for 1 h, then fixed with 4% paraformaldehyde, before being permeabilized with Triton X-100. Then, the samples were placed in 10% goat serum for 2 h, overnight incubated with anti-Iba1 antibody (1:100; Abcam) at 4 °C, then incubated with a FITC-conjugated secondary antibody. The phagocytosed bioparticles were observed using a fluorescence microscope.
2.2.8. Observation of phagocytosis of erythrocytes

After separation and purification of erythrocytes, they were counted and co-cultured with microglia at a ratio of 1:40 (microglia: erythrocytes). Microglia swallowing erythrocytes were observed using a microscope (Olympus CK40–32PH, Japan).

2b. Materials and methods

In our previous study, we found that Nrf2 agonist -monascin facilitates hematoma clearance, alleviates cerebral edema, and exerts neuroprotection after ICH[16,17].

2.1b. Animal materials

Experimental male Nrf2-/-C57BL/6 mice (4–5 weeks old) were purchased from Cyagen Model Biological Research Center (Taicang) Co., Ltd. (Suzhou, China), the other male C57BL/6 mice (4–5 weeks old) were purchased from the Animal Experimental Center of Shanxi Medical University. All animal experiments were approved and conducted as per the guidelines of the Ethics Committee of Shanxi Medical University, Shanxi, China. All surgical procedures were performed under anesthesia, and every effort was expended to minimize suffering.

2.2b. Animal experimental methods

2.2.1. Experimental design

A total of 42 mice were randomized to the following groups: sham (n=8), ICH+vehicle (n =9), Nrf2-/- +ICH(n = 8), ICH+ monascin(10mg/kg/day, twice, n = 8), ICH+ Xuezhikang (0.2g/kg/day, twice, n = 9). Dead animals were replaced before final assessment. All gavages were administered by gastric perfusion 6 h after ICH for 72 hours.

2.2.2. ICH Model

The experimental ICH model was induced by injecting collagenase type IV (0.5 units in 2 μl saline) into the basal ganglia region using stereotaxic instruments. Mice were fixed on a stereotaxic apparatus under a combination of xylazine (10 mg/kg) and ketamine (100 mg/kg); the skull was exposed to reveal bregma. A 1-mm cranial bur hole was drilled in the skull (coordinates: 0.9 mm posterior to the bregma, 1.5 mm lateral to the midline), then, collagenase was infused into the right basal ganglia (4 mm deep from the dura mater) using a microinjector. The needle remained in place for an additional 15 min to prevent “back-leakage”. The Sham-operated mice were syringed with equivalent dosages of physiological saline. After surgery, the skull hole was sealed using bone wax and the incision was sutured. Animals were allowed to recover after successful ICH induction that was confirmed by Rosenberg’s neurological score [40].

2.2.3. Neurobehavior tests

As previously described[6,18-19], before sacrificing the animals for tissue collection, they were subjected to neurofunctional assessments using the modified Garcia tests. Notably, the modified Garcia scale involves an 18-point sensorimotor assessment that includes six individual tests. Each test has a score ranging from 0 to 3, with a maximum score of 18. The individual tests evaluate spontaneous activity, response to side stroking, vibrissae touch, climbing, lateral turning, and forelimb walking.

2.2.4. Immunofluorescence detection
After intraperitoneal anesthesia, heart perfusion was performed using ice Phosphate-buffered saline (PBS) and 4% paraformaldehyde. The brain tissues of mice were removed on ice then placed in a 4% paraformaldehyde overnight at 4°C. Sucrose PBS buffer (20% and 30%) was used for dehydration at 4 °C until tissues were fully penetrated. After fixed embedding of OCT (optimal cutting temperature compound), frozen sections were coronally cut into 4-μm slices. The immunofluorescence methods were based on previously described methods.

2.2.5. Western blot

Total protein from brain tissue was collected, and their concentrations were determined using a BCA kit with a procedure similar to the foregoing cellular experiment methods.

2.3. Statistical analysis

All statistical and graphical analyses and were performed using SPSS 22.0 (IBM, Armonk, NY, USA) and GraphPad Prism 7.0 (https://www.graphpad.com/scientific-software/prism) software. All data were presented as the mean ± SEM (standard error of the mean). One-way analysis of variance (ANOVA) was performed for comparisons among multiple groups, whereas the SNK-q test was used for pairwise comparison between groups. A P-value of less than 0.05 was considered statistically significant.

3. Results

3.1 Mortality, hemoglobin and neurological scores after ICH

All sham-operated mice survived. The total operative mortality rate of mice was estimated at 7.7% (n=2) and was not significantly different among the surgical groups. All ICH groups showed a significant decrease in modified Garcia scores and an increase in hemoglobin levels compared to that of the sham group at 72 hours after surgery (p<0.05, Supplementary Fig A, B). In contrast with the ICH+vehicle, Nrf2 agonist (Monascin and Xuezhikang) treatment significantly improved neurological deficits and reduced hemoglobin levels at 72 hours after ICH; conflicting results were found in Nrf2-/- group (p<0.05, Supplementary Fig. A, B).

3.2 Nrf2 expression

Based on in vitro experiments, Western blot results showed that Nrf2 was greatly improved in the monascin and Xuezhikang groups, while Nrf2 was downregulated in the siRNA group compared to that in the normal control group (Fig. 1 A-C). Furthermore, monascin and Xuezhikang promoted Nrf2 expression in vivo, while they were significantly downregulated in the Nrf2-/- ICH group compared to that in the vehicle ICH group. Consistently, immunofluorescence detection confirmed similar patterns of Nrf2 expression (Fig. 1D-F).

Fig. 1. Immunofluorescence (in vitro×400 and in vivo×200) and Western blot protein expression of Nrf2. Red fluorescence represents Iba1 expression, green fluorescence indicates Nrf2 expression, while blue fluorescence depicts nuclear DAPI staining. “Merge” represents the superposition of the first three images. Western blot analysis and immunofluorescence detection showed that Nrf2 production of microglia was upregulated in the monascin/Xuezhikang groups, while was downregulated in the siRNA group compared to that in the normal group (A)-(C) (**, p< 0.01 vs. the control and *, p< 0.05 vs. the control and WB). Western blot; Nrf2: nuclear factor erythroid 2-related factor 2; Iba1: ionized calcium-binding protein 1, microglial marker). For in vivo experiment, red fluorescence depicts Nrf2 expression, while green fluorescence represents Iba1 expression. Monascin and Xuezhikang promoted Nrf2 expression, while they were significantly downregulated in Nrf2-/- group compared to that in the sham or vehicle group. Representative images of immunofluorescence and Western blot assays are shown (D)-(F) (*, p< 0.05 vs. sham and #, p< 0.05 vs. ICH + vehicle were statistically significant)

3.3 Trem1 and Trem2 protein expression
In the siRNA group, Western blot analysis revealed that the protein levels of Trem2 expression were slightly downregulated, while that of Trem1 expression was upregulated. However, monascin and xuezhikang reversed the protein expression of Trem1 and Trem2 along with Nrf2 upregulation. Immunofluorescence results are similar to that of Western blot (Fig. 2 A-F).

In contrast with the sham group, in vivo Trem1 expression in all the ICH groups was upregulated to varying degrees. Among them, Trem1 expression in the vehicle and Nrf2-/- group was remarkably upregulated. Compared to vehicle group, Trem1 expression was improved in Nrf2-/- group, while that in the monascin and Xuezhikang groups was attenuated. In contrast, the opposite results were observed for Trem2. The immunofluorescence results are roughly consistent with that of Western blot (Fig. 2 G-L).

3.4. Phagocytosis of fluorescent bioparticles

In the phagocytic test of fluorescent bioparticles, the phagocytosis rate of the normal control group was approximately 2-3/cell, that of the siRNA group was about 1-2/cell, whereas that of monascin and Xuezhikang groups (about 4-5/cell) was significantly higher than that of the siRNA group (Fig. 3A).

3.5. Phagocytosis of erythrocytes

In the phagocytic test of erythrocytes, the phagocytosis rate of the normal control group was approximately 1-2/microglial cell, that of the siRNA group was about 0-1/cell, whereas that of monascin and Xuezhikang groups (about 3-6/cell) was significantly higher than that of the siRNA group (Fig. 3B).

3.6. Expression of CD80/CD206

In contrast with the normal group, cellular immunofluorescence demonstrated that both CD80 and CD206 in monascin and Xuezhikang groups were improved when Nrf2 was upregulated; CD206 expression was remarkably upregulated compared to that of CD80. However, CD80 expression in the siRNA group was slightly upregulated, while that of CD206 was downregulated. In this cellular WB, CD206 expression in monascin and Xuezhikang groups was upregulated compared to that in the siRNA group, while that of CD80 was reversed (Fig. 4 A-D).
In vivo experiments, Western blot results revealed that CD80 expression in the four ICH groups was up-regulated compared to that in the sham group. Among them, a significant up-regulation of CD80 was observed in the vehicle or Nrf2-/- group, and no significant difference was noted between the two groups. CD206 expression in the monascin and Xuezhikang groups were significantly up-regulated, whereas CD80 was down-regulated compared with the vehicle group. Immunofluorescence detection results are roughly consistent with that of WB (Fig. 4 E-I).

3.7. Transformation of microglial neuroinflammation

Flow cytometry revealed that the total CD80 expression in the siRNA group was upregulated, the CD206/CD80 ratio was downregulated, while the phenotype was transformed to the pro-inflammatory phenotype. In the monascin and Xuezhikang groups, the total CD206 expression and CD206/CD80 ratio were upregulated, and the microglia were polarized toward the anti-inflammatory phenotype (Fig. 5).

3.8. TNF-α and BDNF protein expression

Nrf2-siRNA promoted the TNF-α protein expression, whereas monascin and Xuezhikang reversed the TNF-α inflammatory factor production instead of BDNF, particularly in the Xuezhikang group (Fig. 6 A-D).

Unlike in the sham or vehicle group, the protein expression of TNF-α in the Nrf2/- group was upregulated, while monascin and Xuezhikang reversed the TNF-α inflammatory factor production instead of BDNF (p< 0.05) (Fig. 6 E-H).

4. Discussion

The present study has made the following observations: (1) The monascin and Xuezhikang groups promote Nrf2 up-regulation in BV-2 microglia cells or C57BL/6 mice; while Nrf2 was down-regulated in the siRNA-Nrf2 group or Nrf2/- group; (2) The monascin and Xuezhikang groups demonstrated a strong capacity to swallow fluorescent bioparticles or erythrocytes, and Trem2,CD206,BDNF expressions were upregulated; (3) On the other hand, Nrf2 inhibition showed conflicting results, i.e., the Trem2,CD206,BDNF expressions were downregulated and the ability to swallow fluorescent bioparticles or erythrocytes was weak. (4) The expression of
Trem1, TNF-α was upregulated in the Nrf2-/- group, while these expressions were reversed to varying degrees in the monascin and Xuezhikang groups compared with the vehicle group. Microglia are the resident macrophages of the central nervous system (CNS) and undergo profound morphological and functional changes when activated. Traditionally, activated microglia initiates an immune response, release inflammatory mediators, and are vital regulators of the neuroinflammatory cascade[18,19]. Alternatively, the activated microglia exerts their anti-inflammatory factors and phagocytic effect via changing their various surface receptors[20]; notably, the local extracellular and intracellular signals determine the features of microglia [21]. Pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) activate microglia to a pro-inflammation state[22]. In neuroinflammatory conditions including ICH and neurodegenerative diseases where pathogenic amyloid-β or hematoma residues accumulate, activation of microglia is promoted primarily via myeloid differentiation factor 88/TIR-domain-containing adaptor inducing interferon-β (MyD88/TRIF). The up-regulation of nuclear factor kappa lightchain enhancer of activated B cells (NF-κB), varying amounts of inflammatory mediators are released, and neuroinflammatory are aggravated, affecting the survival of neuronal cells in the CNS[23,24]. Primary mediators including TNF, IL-1β, and IFNγ are released, promoting the production of secondary mediators, including matrix metalloproteinases (MMP), nitric oxide (NO), and arachidonic acid [25,26]. Besides, TNF-α promotes the production of high-mobility group protein box-1 (HMGB1: a ligand of neuroinflammatory amplifier), which, in turn, stimulates microglia to release large amounts of TNF-α that activate additional microglia [10,27,28]. A self-feedback cascade loop has been reported in the process of microglial activation; these inflammatory signals are amplified by the self-feedback loop of microglial activation constructing an immune cascade inflammation network [18].

Nrf2 is a pleiotropic transcription factor extensively present in microglia. It is a steady-state regulator of cellular oxidative stress with a strong antioxidant capacity. The antioxidants produced, including heme oxygenase (HO-1), superoxide dismutase, catalase, glutathione sulf hydryl transferase, and haptoglobin (Hp) are activated [29]. Moreover, Nrf2 is a vital target that regulates microglia in stroke and neurodegenerative diseases discovered in recent years. Nrf2 upregulation downregulates NF-κB and promotes microglial transformation to the anti-inflammatory phenotype [30,31].

Monascin is a novel type of Nrf2 agonist[32], and an agonist of peroxidase of PPAR-γ, which suppresses oxidative stress in pathological conditions, promotes the absorption of hematoma and reduces intracerebral edema in our animal models of ICH[16,17]. Xuezhikang is an extract of monascin as its primary component; it is primarily used as traditional Chinese medicine to fight atherosclerosis and exert anti-inflammatory effects by inhibiting toll-like receptor 4 (TLR4)/NF-κB[33,34].

Our research found that monascin/Xuezhikang promotes Nrf2 upregulation of Iba1-marked microglia, and Trem2 expression was significantly upregulated correspondingly; besides, their capacity to phagocytize erythrocytes or fluorescent bioparticles was improved after administering Nrf2 agonist. In contrast, Trem2 expression in Nrf2-knocked-down microglia or Nrf2-knocked-out mice was significantly downregulated, and their phagocytic capacity was significantly reduced. Trem2 as a surface receptor of microglia is important to clear or endocytose apoptotic cells and cellular debris, it is essential for neuroplasticity and myelination[35]. It showed that Nrf2 facilitated hematoma clearance via Trem2 on microglia.
Additionally, monascin/Xuezhikang effectively resists the neuroinflammation cascade of Trem1 by up-regulating Nrf2. In all the ICH groups, Trem1 was significantly up-regulated, indicating that Trem1 is highly related to the neuroinflammation of ICH. Trem1 expression in the Nrf2-/- group was significant, while the Nrf2 agonists monascin and Xuezhikang reversed the Trem1 expression. This implies that the change of Nrf2 directly affects the expression of Trem1. In our previous study, we showed that TREM-1 inhibition improves neurological deficits and brain edema in subarachnoid hemorrhage [36]. The microglial cellular experiment further confirmed that Monascin/Xuezhikang effectively suppresses Trem1 expression by upregulating Nrf2. Meanwhile, Trem2 demonstrated beneficial[35]. The mechanism for this phenomenon remains vague, therefore, additional studies on Trem1 and Trem2 are necessary. Here, monascin and Xuezhikang promoted the expression of the anti-inflammatory phenotype marker CD206, and the proportion of CD206/80 was significantly increased. However, the expression of the pro-inflammatory phenotype marker CD80 was upregulated, while that of CD206 was downregulated after Nrf2 was knocked down in microglia or knocked out in mice. These events indicate that the microglia were transformed to anti-inflammatory and phagocytic phenotype after administration of monascin/Xuezhikang. Intriguingly, we found that CD80 also was upregulated in the monascin and Xuezhikang groups in the cellular experiment, however, the upregulation in CD206 was significantly greater than that of CD80, and the ratio of CD206/CD80 was higher. This suggests that monascin and Xuezhikang may strengthen phagocytosis of microglia and are contribute to hematoma clearance. Nonetheless, additional studies are essential to verify efficacy of this. With the transformation of microglia in the anti-inflammatory phenotype, the pro-inflammatory factor TNF-α was significantly decreased instead of the nerve repair factor BDNF, thus significantly inhibiting neuroinflammation. This is consistent with the findings reported in previous studies[37].

To summarize, our findings showed that administration of Nrf2 agonist monascin/Xuezhikang improves neurological deficits in ICH models via microglial phagocytosis and anti-inflammatory by upregulating Trem2, BDNF, and CD206 expressions, while inhibiting the pro-inflammatory cascade of Trem1, TNF-α, and CD80. Microglial functional transformations are implicated in hematoma clearance after ICH.

5. Conclusions

In conclusion, our findings showed that administration of Nrf2 agonist monascin/Xuezhikang improves neurological deficits in ICH models via microglial phagocytosis and anti-inflammation by upregulating Trem2, BDNF, and CD206 expressions, while inhibiting the pro-inflammatory cascade of Trem1, TNF-α, and CD80. Besides, Nrf2 inhibition reversed the effects of Nrf2 agonists. Microglial functional transformations are implicated in hematoma clearance and neuroprotection after ICH. This provided a novel microglia-based approach for the treatment of ICH.

Supplementary Information:

As showed in the supplementary files

Acknowledgments

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Abbreviations

BDNF: Brain-derived neurotrophic factor
CNS: Central nervous system
DAMPs: Danger-associated molecular patterns
HMGB1: High-mobility group protein box-1
HO-1: Heme oxygenase-1
Hp: Haptoglobin
ICH: Intracerebral hemorrhage
IFNγ: Interferon-gamma
IL-1β: Interleukin 1β
MMP: Matrix metalloproteases
Myd88: Myeloid differentiation factor 88
NF-κB: Nuclear factor-kappa lightchain enhancer of activated B cells
NO: Nitric oxide
Nrf2: Nuclear factor erythroid 2-related factor 2
PAMPs: Pathogen-associated molecular patterns
PPAR-γ: Peroxidase proliferator-activated receptor gamma
TCM: Traditional Chinese medicine
TLR4: Toll-like receptor 4
TNFα: Tumor necrosis factor-alpha
Trem1: Triggering receptors I expressed on myeloid cells
Trem2: Triggering receptors II expressed on myeloid cells
TRIF: TIR-domain-containing adaptor inducing interferon-β

Author Contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication. Conceptualization, Gaiqing Wang and John H. Zhang; methodology, Gaiqing Wang and John H. Zhang; validation, Lirong Liu, Shuangjin Bao, Zhenjia Yao, Qinqin Bai, Chuntian Liang, Xiangyu Liu; writing—original draft preparation, Lirong Liu, Shuangjin Bao; writing—review and editing, Gaiqing Wang and John H. Zhang; project administration, Gaiqing Wang, Pengcheng Fu, John H. Zhang; funding acquisition, Gaiqing Wang. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. Please contact the authors of the data and materials.

Ethics approval and consent to participate

All animal experiments were approved and conducted in accordance with the guidelines of the Ethics Committee of Shanxi Medical University, Shanxi, China. All surgical procedures were performed under anesthesia, and every effort was expended to minimize suffering.

Consent for publication

Not applicable.

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

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Immunofluorescence (in vitro×400 and in vivo×200) and Western blot protein expression of Nrf2. Red fluorescence represents Iba1 expression, green fluorescence indicates Nrf2 expression, while blue fluorescence depicts nuclear DAPI staining. “Merge” represents the superposition of the first three images. Western blot analysis and immunofluorescence detection showed that Nrf2 production of microglia was upregulated in the monascin/Xuezhikang groups, while was downregulated in the siRNA group compared to that in the normal group(A)-(C) (*, p< 0.05 vs. the control and **, p< 0.01 vs. the control, WB: Western blot; Nrf2: nuclear factor erythroid 2-related factor 2; Iba1: ionized calcium-binding protein 1, microglial marker). For in vivo experiment, red fluorescence depicts Nrf2 expression, while green fluorescence represents Iba1 expression. Monascin and Xuezhikang promoted Nrf2 expression, while they were
significantly downregulated in Nrf2-/- group compared to that in the sham or vehicle group. Representative images of immunofluorescence and Western blot assays are shown (D)-(F) (*, p< 0.05 vs. sham and #, p< 0.05 vs. ICH + vehicle were statistically significant)
Protein expression of Trem1/Trem2 determined using immunofluorescence (in vitro×400 and in vivo×200) and Western blot. Red fluorescence represents Trem1 or Nrf2 expression, green fluorescence depicts Trem2 expression, while blue fluorescence is nuclear DAPI staining. "Merge" represents the superposition of the first three images. In the siRNA group, immunofluorescence or Western blot results revealed that the protein levels of Trem2 expression were slightly downregulated, while that of Trem1 expression was upregulated. Nonetheless, monascin and Xuezhikang reversed the protein Trem1 and Trem2 expression along with Nrf2 upregulation (A)-(F). Red fluorescence (in vivo experiment) represents Trem1 and Trem2 expression, while green fluorescence is Nrf2 expression. Unlike in the sham group, Trem1 expression in all the ICH groups was up-regulated to varying degrees. In contrast with the vehicle group, Trem1 expression was improved in the Nrf2-/- group, whereas that in monascin and Xuezhikang groups was attenuated. However, Trem2 showed upregulation in monascin and Xuezhikang groups (G)-(L) (Trem1: Triggering receptor I expressed on myeloid cells; Trem2: Triggering receptor II expressed on myeloid cells).

Figure 3

Phagocytosis test of fluorescent bioparticles (immunofluorescence ×100). Green fluorescence is the Iba1 cells, red fluorescence is the fluorescent bioparticles, and blue fluorescence is the nuclear DAPI staining. "Merge" represents the superposition of the first three images. The average phagocytosis of the fluorescent bioparticles was about 2-3/cell in the normal control group, about 1-2/cell in the siRNA group, and 4-5/cell in the monascin/Xuezhikang groups. The phagocytosis rates were significantly increased in the monascin and Xuezhikang groups.

Phagocytosis test of erythrocytes (×400). The white arrow is microglia, the red arrow is erythrocytes, while the blue arrow represents the microglia that swallowed erythrocytes. The average phagocytosis of the erythrocytes was an approximately 1-2/microglial cell in the normal control group, 0-1/cell in the siRNA group, and 3-6/cell in the monascin and Xuezhikang groups. The phagocytosis rates were significantly increased in the monascin and Xuezhikang groups.
Expression of CD80/CD206 detected by immunofluorescence (in vitro×400 and in vivo×200) and Western blot. The green fluorescence represents CD80, the red fluorescence is CD206, while the blue fluorescence is nuclear DAPI staining. “Merge” represents the superposition of the first three images. Unlike in the normal group, cellular immunofluorescence showed that both CD80 and CD206 were improved when Nrf2 was elevated in the monascin and Xuezhikang groups; CD206 expression was significant compared to
that of CD80. In this cellular WB, CD206 expression in monascin and Xuezhikang groups was statistically elevated compared to that in the siRNA group, however, CD80 expression was reversed (A)-(D). For in vivo experiment, the red fluorescence represents CD80, the green fluorescence is CD206. Unlike in the sham group, Western blot analysis revealed that CD80 expression in the four groups of ICH was up-regulated; a significant CD80 upregulation was observed in the vehicle or Nrf2-/- group. CD206 expression in the monascin and Xuezhikang groups was significantly up-regulated, whereas that of CD80 was down-regulated compared with the vehicle group. The results of immunofluorescence detection are roughly consistent with that of WB (E)-(I).
Transformation of microglial neuroinflammation as shown by flow cytometry. The ratio of CD206/CD80 and the total expression of CD206 was significantly upregulated in the monascin and Xuezhikang groups, and the microglial phenotype was transformed to the anti-inflammatory phenotype (green fluorescent FITC: CD80; red fluorescent APC: CD206).

Figure 6
Protein expression of inflammatory factor TNF-α and BDNF by Western blot. The protein expression of BDNF was significantly upregulated in the monascin and Xuezhikang groups; however, it was downregulated in the Nrf2- siRNA group(A)-(D) or in the Nrf2-/- group(E)-(H) TNF-α protein expression downregulated in the monascin and Xuezhikang groups while it was upregulated in the Nrf2-siRNA or Nrf2-/- group (TNF-α: tumor necrosis factor α; BDNF: brain-derived growth factor)

**Supplementary Files**

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- SupplementalFig.docx