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Original Research

Matrine alleviates early brain injury after experimental subarachnoid hemorrhage in rats: possible involvement of PI3K/Akt-mediated NF-κB inhibition and Keap1/Nrf2-dependent HO-1 induction

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Abstract: Matrine is a quinolizidine alkaloid derived from the herb *Radix Sophorae Flavescentis*, and possesses anti-oxidant, anti-inflammatory and anti-tumoral properties. However, its effectiveness against subarachnoid hemorrhage (SAH) is not well known. In this study, we investigated the effects of matrine on early brain injury (EBI) and the related potential mechanisms following SAH in rats. Our results showed that matrine pretreatment partially alleviated SAH-induced EBI, including neurological deficit, severity of SAH grade, brain edema, and blood-brain barrier (BBB) disruption in rats. In addition, SAH procedure induced BBB disruption with concomitant upregulation of MMP-9 expression and downregulation of tight junction proteins expression of BBB, namely, ZO-1 and occludin, which was partially reversed by matrine pretreatment. Matrine also reduced the increased levels of inflammatory cytokines TNF-α and IL-1β after the SAH operation. SAH induced neural cell apoptosis, as demonstrated by high apoptotic index and increased expression of Bax and caspase-3 proteins, as well as the reduced Bcl-2 expression, which were reversed by matrine pretreatment. Furthermore, matrine pretreatment partially suppressed SAH-induced Akt phosphorylation and IκB-α phosphorylation and degradation, and reduced NF-kB P65 protein levels. The expression of Keap1, Nrf2, and HO-1 proteins was distinctly enhanced in the SAH+matrine group, compared with the SAH+vehicle groups. Matrine pretreatment suppressed SAH-induced MMP-9 expression, which could be partially blocked by HO-1 inhibitor Sn-protoporphyrin IX (SnPP IX) but promoted by phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002. Our results suggest that matrine may alleviate EBI after experimental subarachnoid hemorrhage in rats possibly via PI3K/Akt-mediated NF-κB inhibition and Keap1/Nrf2-dependent HO-1 induction.

Key words: Matrine, subarachnoid hemorrhage, blood-brain barrier disruption, early brain injury, NF-κB, HO-1.

Introduction

Subarachnoid hemorrhage (SAH) is a fatal subtype of stroke that accounts for 5–7% of all strokes, despite recent therapeutic advances (1, 2). Early brain injury (EBI) seems to be the primary cause of high mortality and morbidity for patients suffering from SAH (3). The complex pathophysiological process of EBI involves several possible mechanisms, such as rapidly rising intracranial pressure, reduced cerebral perfusion pressure, blood–brain barrier (BBB) disruption, and peripheral immune cells infiltrate into the brain, leading to increased production of several inflammatory cytokines and chemokines, and thus brain edema (2, 4, 5). Brain edema is a typical feature of experimental and clinical SAH that results from BBB disruption (6, 7). Thus, BBB disruption plays a crucial role in the development of EBI after SAH, and it is essential to develop new therapies against BBB disruption and EBI for amelioration of the poor outcomes among SAH patients.

Matrix metalloproteinases (MMPs) have been implicated in the process of BBB disruption (8). Matrix metalloproteinase-9 (MMP-9) belongs to the MMPs family of zinc-containing proteinases and is a key mediator of BBB disruption in central nervous system (CNS) diseases, including SAH (9, 10). MMP-9 upregulation after the onset of SAH has been associated with the disruption of BBB permeability (9). Early blockade of MMP-9 expression and activity stabilizes the BBB permeability and confers early neuroprotection (11, 12), which may be a potential therapeutic strategy for SAH.

Accumulating evidence suggests that active ingredients from natural products can be used to prevent and treat SAH. These products include apigenin (13), resveratrol (14), and curcumin (15), and involve many advantages such as abundant resources, multi-targeted mechanisms of activity, few side effects, and no drug resistance. Matrine ($C_{15}H_{24}N_2O$) is a natural compound derived from the herb *Radix Sophorae Flavescentis*. It has been suggested to possess a wide spectrum of biological activities, including anti-viral (16), anti-oxidant (17), anti-inflammatory (18), anti-fibrotic (19), and anti-tumoral (20) properties. Recently, it has been reported that matrine suppresses focal cerebral ischemic injury through its anti-oxidant and anti-apoptotic properties (21). Matrine treatment also attenuates MMP-9 expression, leading to reduced BBB leakage in the rat model of experimental autoimmune encephalomyelitis (EAE) (22). Thus, this research currently aimed to investigate the neuroprotective effects of matrine on EBI following SAH, and to further explore the related mechanisms in rats.

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Materials and Methods

Animal preparation

Male Sprague-Dawley (SD) rats, weighing 300 to 350 g, were purchased from the Animal Center of Chinese Academy of Sciences (Shanghai, China). The rats were housed in temperature- and humidity-controlled animal quarters with a 12-h light/dark cycle and fed a standard pellet chow and water ad libitum. All procedures involving animals complied with the Guide for the Care and Use of Laboratory Animal published by the US National Institutes of Health (NIH Publication, 1996) and were approved by the Institutional Animal Use and Care Committee of the Medical School (Xi'an Jiaotong University, China).

Experimental SAH rat model

The experimental SAH model was established using stereotaxic insertion of a needle into the prechiasmatic cistern according to a previous study (23). The rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg; Fort Dodge Laboratories, Inc., Fort Dodge, IA) and secured in a stereotactic frame. Then, 0.3 mL of non-heparinized fresh autologous arterial blood was withdrawn from the unilateral femoral artery. The blood was slowly injected into the prechiasmatic cistern over 20 s with a syringe pump using an aseptic technique. Rats in the control group were injected with 0.3 mL of saline solution. After the operation, 1 mL of 0.9% NaCl solution was injected subcutaneously to prevent dehydration, and rats were kept in a 30° head-down position for 20 min. After recovery from anesthesia, the rats were returned to their cages.

Experimental design and drug pretreatment

The rats were randomly divided into five groups as follows: the control group (n=24), the SAH group (n=24), the SAH+vehicle group (n=24), and the SAH+matrine group (n=24). The matrine (purity ≥98.0%; Jiangsu Chia Tai Tianqing Pharmaceutical Co., Jiangsu, China) was dissolved in normal saline solution and injected intraperitoneally daily at a dosage of 250 mg/kg, which was calculated at 6.7 mL/kg (22). Matrine was administered once daily via intraperitoneal injection prior to testing for 7 consecutive days and 0.5 h after the SAH operation to maintain drug levels. The vehicle-treated group received an intraperitoneal saline injection instead of matrine.

To study the mechanisms, phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (Cell Signaling Technology, Beverly, MA, USA) and HO inhibitor Sn-protoporphyrin IX (SnPP IX; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for intervention; namely, the SAH+LY294002 group (n=6), and the SAH+SnPP IX group (n=6), the SAH+matrine+LY294002 group (n=6), and the SAH+matrine+SnPP IX group (n=6), which were specially used for protein extraction. Thirty minutes after SAH, 10 μL LY294002 (50 mM), 10 μL SnPP IX solution (3.3 μg), or 10 μL vehicle, respectively, were injected intracerebroventricularly (bregma: 1.4 mm lateral, 0.8 mm posterior, 3.6 mm deep) by a pump at a rate of 0.5 μL/min, as described previously (24).

After SAH induction for 24 h, neurological scores were evaluated. The rats were then decapitated: a quarter from each group were decapitated for the Evans blue (EB) assay, a quarter for evaluation of SAH severity and brain edema, a quarter for the ELISA and western blot analyses, and a quarter received fixative perfusion for TUNEL staining analysis.

Assay of neurological score

Neurological scores were randomly evaluated using a Garcia scoring system (25). The evaluation comprised six tests with scores of 0–3 or 1–3: spontaneous activity (0–3), symmetry movements of the four limbs (0–3), forepaw outstretching (0–3), climbing (1–3), body proprioception (1–3), and response to vibrissae stimulus (1–3) as showed in Table 1. Clinical scores were recorded by an individual who was blinded to the experimental groupings. The maximum score was 18 and the minimum was 3. A higher score indicated greater neurologic function.

Evaluation of SAH severity

After the rats were sacrificed, the severity of SAH was quantified by Sugawara's grading scale (26). The basal cistern was divided into six parts, depending on the amount of subarachnoid blood clot, and alloted a grade from 0 to 3 as follows: Grade 0: no subarach-

Table 1. Neurological evaluation after SAH treatment in rats.

Test	score			
	0	1	2	3
Spontaneous activity (in cage for 5 min)	No movement	Bare moves	Moves but does not approach at least three sides of cage	Moves and approaches at least three sides of cage
Symmetry movements of the four limbs	Left side: no movement	Left side: slight movement	Left side: moves slowly	Both sides: move symmetrically
Forepaw outstretching	Left side: no movement, no outreaching	Left side: slight movement to outreach	Left side: moves and outreaches less than right side	Symmetrical outreach
Climbing	…	Fails to climb	Left side is weak	Normal climbing
Body proprioception	…	No response on left side	Weak response on left side	Symmetrical response
Response to vibrissae stimulus	…	No response on left side	Weak response on left side	Symmetrical response

noid blood, 1: minimal subarachnoid blood, 2: moderate blood clot with recognizable arteries, 3: blood clot obliterating all arteries within the segment (total score: 0–18). In this method, the arteries within the basal cistern were included and used for this grading system to maintain reproducibility and consistency.

Evaluation of the brain edema

After the rats were sacrificed, the brain water content was evaluated as previously described (11). The brains were removed and weighed immediately as wet weight. Brain specimens were dried in an oven at 80°C for 72 h and weighed again as dry weight. The percentage of water content was calculated as ((wet weight − dry weight)/wet weigh)×100%.

Test of BBB permeability

BBB permeability was quantitatively evaluated by EB extravasation. Briefly, EB dye (2%, 5 ml/kg; Sigma, St. Louis, MO, USA) was injected into the left femoral vein and allowed to circulate for 1 h. Under deep anesthesia, rats were sacrificed by intracardial perfusion with saline. The brains were then removed, weighed, and immersed in formamide (10 mL/g), and then incubated at 60°C for 24 h. The extravasations were measured for the absorbance of EB at 620 nm using a spectrophotometer.

Enzyme-linked immunosorbent assays (ELISA) assay

Cell lysates were collected and protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The levels of inflammatory cytokines in the brain tissue were quantified using commercial ELISA kits specific for rats, according to the manufacturers' instructions (TNF-α, Diaclone Research, Cedex, France; IL-1β, Biosource Europe SA, Nivelles, Belgium). The cytokine contents in the brain tissue were expressed as picogram per milligram protein.

TUNEL staining assay

The TUNEL staining was performed to detect the apoptotic cells using the TUNEL Staining Kit (Roche Inc, Basel, Switzerland). After decapitation, the rats were perfused intracardially first with phosphate buffer saline (PBS; pH 7.4), and then with 4% paraformaldehyde (pH 7.4). The brains were collected, placed at 4°C in the same fixative for 7 d, and then embedded in paraffin and cut into 10-μm slices. The brain sections were processed by TUNEL staining according to the manufacturer's protocol. The extent of brain damage was evaluated by the apoptotic index, which represented the average percentage of TUNEL-positive cells in each region counted in six fields. The apoptotic index was calculated according to the following formula: apoptotic index (%) = (apoptotic cell number/total cell number)×100.

Western blot analysis

Equal amounts of protein were processed for western blotting following the standard protocols. The primary antibodies used were anti-p-Akt, anti-Akt, and anti-caspase-3 antibodies (Cell Signaling Technology, Beverly, MA, USA); anti-ZO-1, anti-occludin, anti-Bax, anti-Bcl-2, anti-NF-κB P65, anti-IκB-α, anti-p-IκB-α, anti-Kelch-like ECH-associating protein 1 (Keap1), anti-nuclear factor-erythroid related factor-2 (Nrf2), anti-heme oxygenase 1 (HO-1), and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and anti-MMP-9 antibody (Abcam, San Francisco, CA, USA). The resultant protein bands after incubation with proper secondary antibody were visualized by electrochemiluminescence (ECL; Beyotime, Shanghai, China). The absorbance values of the target proteins were performed through Gel-Pro Analyzer version 4.0 software (Media Cybernetics, Silver Spring, MD, USA).

NF-κB assay

The NF-κB activity in nuclear extracts was analyzed by a NF-κB p65 ActivELISA kit (Imgenex, San Diego, CA, USA) according to the manufacturer's instructions. The absorbance was determined using a microplate reader set at 405 nm.

Statistical analysis

All data were expressed as mean±SD of results derived from three independent experiments performed in triplicate. All analyses were conducted using SPSS software (SPSS Inc., Chicago, IL, USA). Difference comparison was performed with one-way ANOVA analysis followed by post-hoc tests. $P<0.05$ were considered statistically significant compared to the respective control.

Results

Matrine partially alleviated EBI caused by SAH

After 24 h of SAH, a series of physiological indices was tested to evaluate the effects of matrine on EBI in the rat SAH model. First, the neurological scores of the SAH and SAH+vehicle groups were significantly lower than that of the control group, whereas matrine pretreatment improved the neurological scores (Fig. 1A). We then observed the higher SAH grading scores in the SAH and SAH+vehicle groups than in the control group, which were reduced by matrine pretreatment (Fig. 1B). At the same time, the brain water content levels in the SAH group and SAH+vehicle group were significantly increased, compared to the control group, and matrine attenuated the increase in brain water content induced by SAH (Fig. 1C). These data indicated that SAH induced EBI accompanied by reduced neurological scores, increased SAH grading scores, and brain edema. These outcomes were partially reversed by matrine pretreatment, suggesting that matrine may have a neuroprotective effect on SAH-induced EBI in rats.

Matrine partially ameliorated BBB disruption induced by SAH

In our study, BBB permeability was tested 24 h after SAH. The amount of extravasated Evans blue dye in the brain was significantly elevated in the SAH group, compared to that of the control group. Matrine pretreatment resulted in decreased dye extravasation into the brain, compared to the SAH+vehicle group (Fig. 2A). Then we examined the effects of matrine on the tight junction proteins expression of BBB permeability, namely,

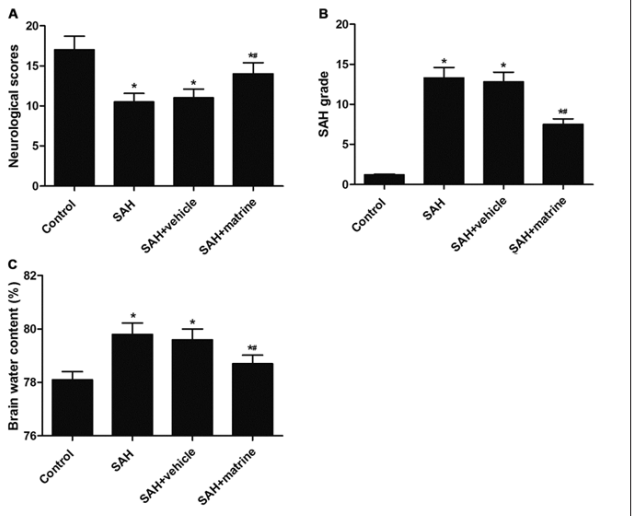

Figure 1. Effect of matrine on EBI at 24 h after SAH in rats. The physiological indices of neurological scores (A), SAH grade (B), and brain water content (C) in each group were determined at 24 h after SAH. **P*<0.05 *vs.* control group; #*P*<0.05 *vs.* SAH+vehicle group (n=6).

MMP-9, ZO-1, and occludin, respectively. The upregulation of MMP-9 was observed in SAH-treated rats, which was then restored by matrine pretreatment. The protein levels of ZO-1 and occludin were dramatically degraded following SAH, and matrine pretreatment reduced the degradation, compared to the SAH+vehicle group (Fig. 2B). These results indicated that matrine partially ameliorated SAH-induced BBB disruption by downregulating the MMP-9 expression and upregulating the tight junction proteins expression of BBB.

Matrine decreased production of pro-inflammatory cytokines induced by SAH

Levels of pro-inflammatory cytokines TNF-α and IL-1β were detected by commercial ELISA kits. The levels of TNF-α and IL-1β were enhanced after the SAH operation and then attenuated by matrine pretreatment (Fig. 3). These results revealed that matrine reduced the inflammation response induced by SAH operation in rats.

Matrine suppressed neural cell apoptosis induced by SAH

We observed the number of TUNEL-positive cells in the rat brains. Compared to the control group, a higher apoptotic index was found in the SAH group. However, matrine pretreatment decreased neuronal apoptosis, as demonstrated by the low apoptotic index in the SAH+matrine group compared with the SAH+vehicle group (Fig. 4A). The expression of Bax and caspase-3 proteins was increased and Bcl-2 expression was reduced in the SAH and the SAH+vehicle groups, which were reversed by matrine pretreatment (Fig. 4B). These results indicated that matrine pretreatment suppressed SAH-induced neural cell apoptosis in rats.

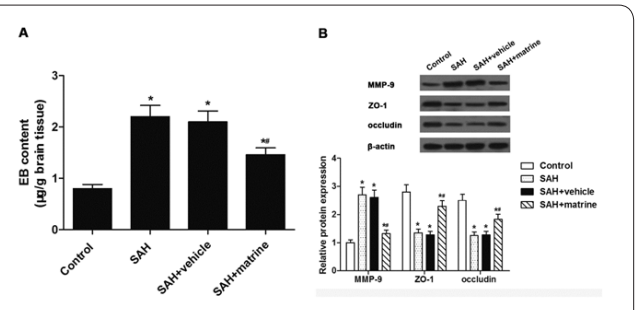

Figure 2. Effect of matrine on BBB disruption and expression of BBB-related proteins at 24 h after SAH in rats. (A) The BBB permeability was quantitatively evaluated by EB content using a spectrophotometer. (B) The expression of MMP-9, ZO-1, and occludin proteins was determined by western blotting and representative blots are shown. **P*<0.05 *vs.* control group; #*P*<0.05 *vs.* SAH+vehicle group (n=6).

Both PI3K/Akt-mediated NF-κB inhibition and Keap1/Nrf2-dependent HO-1 induction were possibly involved in matrine-inhibited MMP-9 expression

Western blot revealed that matrine pretreatment suppressed SAH-induced Akt phosphorylation and IκB-α phosphorylation and degradation, and reduced protein levels of NF-kB P65 (Fig. 5A). Matrine clearly suppressed the constitutive and inducible NF-κB activity caused by SAH (Fig. 5B), which could be concluded that SAH-induced translocation of the NF-κB p65 subunit to the nucleus could be inhibited by matrine to some extent. SAH slightly increased the low levels of Keap1, Nrf2, and HO-1 proteins, which were distinctly en-

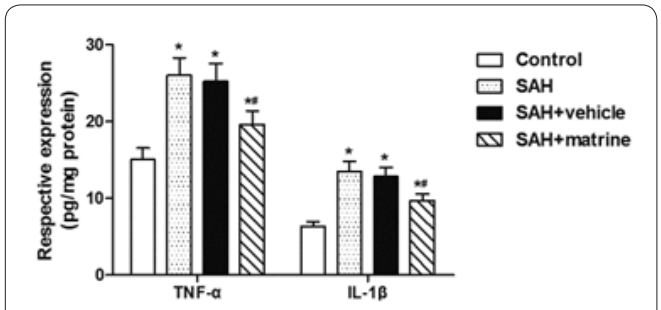

Figure 3. Effect of matrine on inflammatory cytokines expression at 24 h after SAH in rats. The levels of TNF-α and IL-1β were quantified using commercial ELISA kits. **P*<0.05 *vs.* control group; #*P*<0.05 *vs.* SAH+vehicle group (n=6).

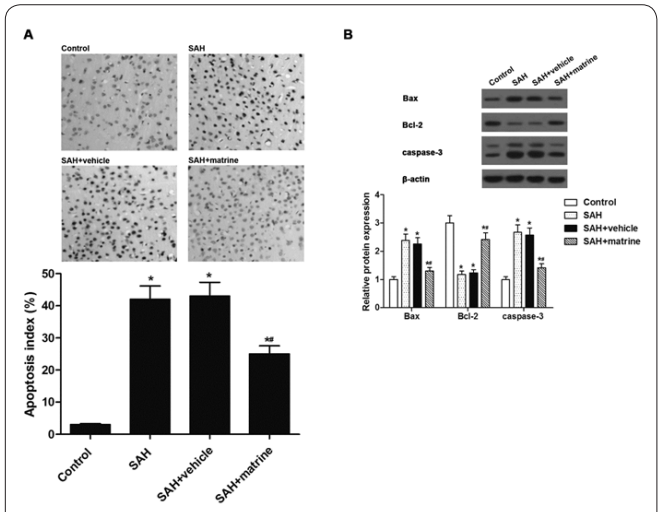

Figure 4. Effects of matrine on neuronal cell apoptosis at 24 h after SAH in rats. (A) Cell apoptosis was detected using the TUNEL Staining Kit and the apoptotic index was calculated as indicated. (B) The expression of Bax, Bcl-2 and caspase-3 proteins was determined by western blotting and representative blots are shown. **P*<0.05 *vs.* control group; #*P*<0.05 *vs.* SAH+vehicle group (n=6).

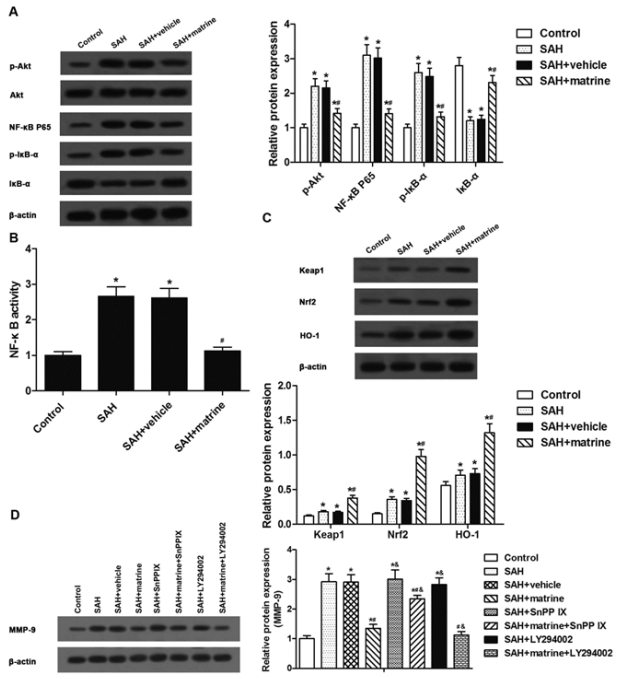

Figure 5. Effects of matrine on PI3K/Akt-mediated NF-κB and Keap1/Nrf-2/HO-1 signaling pathways and the expression of MMP-9 at 24 h after SAH in rats. (B) The NF-κB activity was detected by a NF-κB p65 ActivELISA kit. (A, C, D) The expression of p-Akt, Akt, NF-κB P65, p-IκB-α, IκB-α, Keap1, Nrf2, HO-1, MMP-9, and β-actin proteins was determined by western blotting and representative blots are shown. **P*<0.05 *vs.* control group; #*P*<0.05 *vs.* SAH+vehicle group; &*P*<0.05 vs. SAH+matrine group (n=6).

hanced by matrine pretreatment (Fig. 5C). Furthermore, matrine pretreatment suppressed SAH-induced MMP-9 expression, which was partially reversed by SnPP IX but promoted by LY294002. LY294002 aggravated the inhibition of matrine on MMP-9 expression after SAH in some degree, whereas SnPP IX acted inversely (Fig. 5D). These data suggest that both PI3K/Akt-mediated NF-κB inhibition and Keap1/Nrf2-dependent HO-1 induction were possibly involved in matrine-inhibited MMP-9 expression in SAH-treated rats.

Discussion

Clinically, SAH is a severe disease associated with high mortality (3). Studies have indicated that EBI, which occurs within the first 72 h after SAH, is a primary factor that contributes to poor outcomes for patients with SAH (2, 5). Matrine is the main alkaloid extracted from the traditional Chinese herb. It has been demonstrated to possess a variety of pharmacological activities, especially anti-oxidant (17) and anti-inflammatory (18) properties. In the present study, we mainly focused on the effects of matrine on EBI and the potential mechanisms responsible for the neuroprotective effects of matrine following SAH in rats.

Recently, EBI has emerged as a new frontier and thus requires a better understanding and consideration in devising therapeutic strategies for ameliorating SAH outcomes. Previous studies have confirmed that SAH can induce EBI, including neurological deficit, severity of SAH grade, brain edema, BBB disruption, and neural cell apoptosis in rats (13, 27, 28). Matrine is an active constituent that has beneficial effects against focal cerebral ischemia (29). Pretreatment with matrine significantly decreases the infarct volume and improves the neurological scores in a mouse model of focal cerebral ischemic injury (21). In this study, the results indicated that SAH induced EBI accompanied by reduced neurological scores, increased SAH grading scores, enhanced brain edema levels, and increased BBB disruption in rats, which were partially alleviated by matrine pretreatment. Matrine may thus have a neuroprotective effect on SAH-induced EBI in rats.

It is known that MMPs, especially MMP-9, play a crucial role in the pathogenesis of secondary brain injury after SAH. MMP-9 upregulation has been observed to be involved in EBI after SAH in the cerebral cortex, and this EBI can be reduced by MMP-9 inhibition (12). The downregulation of tight junction proteins, including Zonula occludens-1 (ZO-1) and occludin following SAH, has been closely related to the disruption of BBB integrity (13). Preventing disruption of tight junction proteins of BBB may play a role in attenuating brain edema secondary to BBB dysfunctions (30). In addition, MMP-9 can degrade interendothelial tight junction proteins and basal lamina proteins of the BBB, leading to BBB breakdown (27). Our results showed that SAH treatment induced BBB disruption with concomitant MMP-9 upregulation and downregulation of the tight junction proteins expression of BBB, including ZO-1 and occluding, which were partially reversed by matrine pretreatment.

Following SAH, the inflammatory response is accompanied by mass production of inflammatory cytokines, resulting in brain edema and neuronal injury (27). Both the number of TUNEL-positive neurons in the cerebral cortex and the levels of pro-inflammatory cytokines IL-1β and TNF-α were increased in EBI after SAH (12, 30). Matrine effectively protects neuroaxons from CNS damage caused by inflammation in the rat EAE model (31). Matrine pretreatment induced a marked decrease in caspase-3 expression and an increase in the Bcl-2/Bax ratio in a mouse model of focal cerebral ischemic injury (21). Similarly, we observed that TNF-α and IL-1β levels, and neural cell apoptosis were enhanced after the SAH operation and then partially restored by matrine pretreatment. Morover, the enhancement of BBB permeability and neural cell death has been associated with the remarkable elevation of IκB-α degradation, NF-κB translocation to nucleus, as well as MMP-9 expression, tight junctions protein, IL-1β and TNF-α in SAH rats (27). Attenuation of SAH-induced cortical neuronal apoptosis is probably mediated by activation of Keap1/Nrf2/ARE pathway (32). Thus, we further explored whether NF-κB and Keap1/Nrf2/ARE signaling pathways were involved in the effects of matrine on EBI in the rat model of SAH.

Previous study has revealed that SAH induced the activation of NF-κB, thus promoting the translocation of p65 subunits into the nucleus and increasing the mRNA levels of its downstream pro-inflammatory cytokines IL-1β and TNF-α (33). NF-κB accelerates the inflammatory cascade in different cell types following SAH, and it also regulates the transcription of pro-inflammatory proteins, including TNF-α (34) and MMP-

9 (11). Enhanced PI3K/Akt phosphorylation is revealed as a main upstream pathway of increased MMP-9 expression accompanying NF-κB activation, which can be diminished by LY294002 (35). In addition, MMP-9 inhibition has been linked with NF-κB inactivation and Nrf2-mediated HO-1 induction (36). HO-1 induction is partially involved in the inhibition of MMP-9 activation in MCF-7 cells (37). We speculated that inhibition of Keap1/Nrf2-dependent HO-1 induction may counteract matrine-inhibited MMP-9 expression, and inhibition of PI3K/Akt-mediated NF-κB activity may contribute to matrine-inhibited MMP-9 expression. Our data revealed that both PI3K/Akt-mediated NF-κB inhibition and Keap1/Nrf2-dependent HO-1 induction were possibly involved in matrine-inhibited MMP-9 expression. LY294002 aggravated the inhibitory of matrine on MMP-9 expression after SAH in some degree, whereas SnPP IX acted inversely. Moreover, matrine pretreatment distinctly enhanced the slight increase of Nrf2 protein levels in SAH, accompanied by increased levels of regulatory protein Keap1, which is consistent with previous researches (32, 38). However, the concrete mechanism underlying the role of Keap1/Nrf2-dependent signaling pathway after SAH still needs further research.

Taken together, these results suggest that matrine may alleviate EBI after experimental subarachnoid hemorrhage in rats possibly via PI3K/Akt-mediated NF-κB inhibition and Keap1/Nrf2-dependent HO-1 induction. The findings further support that matrine might be a potential candidate agent against EBI for SAH patients.

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