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Monomethyl fumarate protects cerebral hemorrhage injury in rats via activating microRNA-139/Nrf2 axis

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Abstract. – OBJECTIVE: Monomethyl fumarate (MF) exerts anti-inflammatory and antioxidant capacities. Whether microRNA-139 and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) are involved in the pharmacological activity of MF remain unclear. We aim to elucidate the potential function of MF in intracerebral hemorrhage (ICH), and its possible mechanism.

MATERIALS AND METHODS: Twenty-four Sprague Dawley (SD) rats were randomly assigned into sham group, ICH group and MF group, with 8 rats in each group. Rats in ICH and MF group were subjected to ICH procedures. Rat brain tissues were harvested at 48 h after ICH procedures. Evans blue extravasation was performed to evaluate ICH-induced rat brain damage. Content of cerebral edema and neurological deficit were examined to reflect the neuronal pathological lesions. Reactive oxygen species (ROS) content in rat brain was examined by immunofluorescence. Activities of oxidative stress indexes in rat brain homogenate were detected using relative commercial kits. MicroRNA-139 expression in rat brain was quantified by quantitative Real-time polymerase chain reaction (qRT-PCR). Finally, protein levels of Nrf2, HO-1, NQO1 and nuclear factor-kappa B (NF-κB) in rat brain tissues were examined by Western blot.

RESULTS: Compared with rats in sham group, neurological deficit scores of rats in ICH group were lower. Disruption of blood-brain barrier and brain tissue edema of rats were pronounced in ICH group. However, MF pretreatment markedly alleviated the above mentioned cerebral lesions. In addition, MF pretreatment increased activities of SOD, GSH and CAT, but decreased MDA and ROS contents in rat brain homogenate relative to those in ICH group ($p<0.05$). Western blot analysis found that expression levels of Nrf2, HO-1 and NQO-1 were markedly upregulated after MF pretreatment, while the expression level of NF-κB was downregulated. At the cellular level, we altered microRNA-139 expression in SH-SY5Y cells by transfection of microRNA-139 mimics or inhibitor. Overexpression of microRNA-139 remarkably increased Nrf2 expression and decreased NF-κB expression. Treatment of high-dose MF upregulated Nrf2, downregulated NF-κB and decreased ROS content in SH-SY5Y cells.

CONCLUSIONS: MF protects ICH in rats by inhibiting oxidative stress and inflammatory response through activating microRNA-139/Nrf2 axis.

Key Words:
Monomethyl fumarate, Nrf2, MicroRNA-139, ICH.

Introduction

Intracerebral hemorrhage (ICH) is a common and frequently occurring disease that seriously endangers the elderly. Its incidence is about 20-30% in stroke patients, which is the leading cause of stroke-induced death[1-3]. A series of complex pathophysiological changes emerge in the brain at post-ICH, such as cerebral edema, inflammatory reaction, energy metabolism disorder and oxygen free radical damage[1,2,4]. Current clinical treatment for ICH is mainly based on symptomatic treatments, including dehydration alleviation, intracranial pressure reduction, blood pressure stabilization, and improvement of brain nutrition metabolism and complication prevention. Surgery is preferred in ICH patients with a large amount of hemorrhage and surgical indication[5,6]. Pathological mechanism of ICH is complex, and its mortality and disability still remain high. Survived ICH patients often experience neurological dysfunction[7-9]. Currently, it is believed that neutrophil and macrophage infiltration, microglia activation, and inflammatory response are involved in the pathogenesis of ICH[4,6,7]. MicroRNAs are a recently discovered non-coding RNAs consisting of 22 nucleotides[10,11]. MicroRNAs

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were first identified in the pathological process of tumors, and they have been proved to participate in cellular physiological and pathological processes as well[11]. MicroRNA-139 is closely related to the damage repair of the body[12,13]. As one of the effective oxidative stress pathways in the body, nuclear factor (erythroid-derived 2)-like 2-antioxidant response element (Nrf2-ARE) pathway is capable of reducing oxidative stress and cytotoxicity[14]. Once Nrf2-ARE pathway is activated, it upregulates expressions of detoxifying and antioxidant enzymes. Nrf2-ARE pathway helps to stabilize the intracellular environment, scavenge oxygen free radicals and prevent oxidative stress[14-16]. Nrf2 induces coordinated expressions of the phase II enzymes after binding to the enhancer sequence of ARE[16]. It eliminates ROS/RNS-induced cell damage, endogenous and exogenous chemicals, showing a crucial role in cell defense protection[16,17]. Monomethyl fumarate (MF) was originally identified as a very effective radiosensitizer for hypoxic cells[18]. Studies have found that MF can be applied for oral treatment of psoriasis, annular atrophic plaques, *granuloma annulare*, sarcoidosis and other traumatic diseases[18,19]. Recently, clinical trials have found that MF can successfully reduce the recurrent rate and time of disability progression in multiple sclerosis by activating Nrf2-ARE pathway[20]. More importantly, MF dose not lead to immunosuppression[18, 19]. In this paper, we detected the *in vivo* and *in vitro* effects of MF on ICH-induced brain damage. Our study provides a solid foundation for clinical application of MF in the treatment and prevention of ICH.

Materials and Methods

Cell Culture

Human-derived SH-SY5Y cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). High-glucose Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) was used for cell culture at 37°C, 5% CO_2.

Establishment of ICH Model in Rats

Adult Sprague Dawley (SD) rats weighing 200±20 g were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Experimental animals were housed in a standard environment with free access to drink and food.

The ICH model was established by injecting autologous blood into the rat anterior chiasmatic cistern. After anesthesia with intraperitoneal injection of 10% chloral hydrate (4 mL/kg), rats were fixed on the operation table in the prone position. A 1-cm incision was made alongside the median sagittal line to bluntly separate and expose the skull. At 7.5 mm anterior to the bregma and 3 mm beside the midline, the bone window (1.5-2 mm in diameter) was opened by dental electric drilling. Compression hemostasis was applied if necessary. Rats were then turned over in a supine position. An incision was made at the inguinal ligament to separate the femoral artery. We extracted 0.35 mL of femoral artery blood. Subsequently, a syringe containing 0.3 mL of non-coagulated femoral artery blood was inserted 10-12 mm into the anterior chiasmatic cistern at a 45-degree angle from the sagittal plane within 15-20 s. 10 mL of normal saline was subcutaneously injected in the back of the rat to prevent postoperative dehydration. Rat lower limbs were kept higher than its head for half an hour, and individually housed after ICH procedures. A total of 24 rats were randomly assigned into sham group (opening of bone window without injecting artery blood), ICH group and MF group (MF treatment after ICH procedures), with 8 in each group. For rats in MF group, rats received intragastric administration of 15 mg/kg MF treatment at 1 h, 12 h, 24 h and 36 h after ICH procedure. Rat activity was regularly observed.

Evans Blue Extravasation

At 48 h of post-ICH, rats were anesthetized with 10% chloral hydrate. 2% Evans blue (2 mL/kg) was intravenously injected into the femoral vein or vena epigastrica. After 1 h, the left ventricle was perfused at 100 cm H_2O pressure until the systemic blood was discharged. The cranial cortex was immediately harvested for analyzing blood-brain barrier dysfunction.

Evaluation of Neurological Deficits

At 48 h of post-ICH, rat neurological deficits were evaluated by the Yamaguchi neurological scoring system. Activity, food intake and functional impairment were evaluated. Higher scores indicated worse neurological deficits.

Analysis of Cerebral Edema Index

Rats were anesthetized with 10% chloral hydrate at 48 h after ICH procedures. The cranial cortex around the blood clot was harvested and

weighed, which was recorded as the wet weight. Tissues were placed in an incubator (80°C) for 48 h and then weighed twice, which was recorded as dry weight. The difference between the two measurements was <0.2 mg. Cerebral edema index = (wet weight - dry weight) / wet weight × 100%.

Cell Transfection

SH-SY5Y cells were transfected with miRNA-NC, miRNA-139 inhibitor, miRNA-139 mimics or negative control using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). At 48 h of transfection, relative genes in transfected cells were determined by quantitative Real-time polymerase chain reaction (qRT-PCR).

Cell Proliferation Assay

Pre-treated cells were digested and seeded into 96-well plates (1×10^4/well). 24 h later, 10 μL of cell counting kit-8 (CCK-8) reagent (Dojindo, Kumamoto, Japan) was added in each well. Cells were then gently mixed and continued to incubate for 2 h at 37°C. The absorbance (OD value) at 450 nm was detected by a microplate reader.

Biochemical Measurements

After ICH procedures, rats were sacrificed for harvesting serum samples. Activities of malondialdehyde (MDA), catalase (CAT), reduced glutathione (GSH), superoxide dismutase (SOD) and reactive oxygen species (ROS) were quantified using relative commercial kits (Nanjing Jiancheng Biotechnology Co. Ltd. Nanjing, China).

QRT-PCR

Total RNA was extracted from cells or tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). 1 μg of total RNA was subjected to reverse transcription into cDNA using the Primescript RT kit (TaKaRa Bio Inc., Otsu, Shiga, Japan). QRT-PCR was performed using the SYBR premix Ex TaqII kit (TaKaRa, Otsu, Shiga, Japan) on an ABI Prism 7500 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). U6 was utilized as an internal reference. Primer sequences were as follows: MiRNA-139, forward: 5'-AGCGTTCTACAGTGCACGTG-3', reverse: 5'-GTGCAGGGTCCGAGGT-3'; U6, forward: 5'-CGCAAGGATGACACGCAAATTC-3', reverse: 5'-TATATCACTCTTGCTTCA-3'.

Western Blot

Total protein from cells or tissues was extracted using radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) and loaded for electrophoresis. After transferring on a polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland) at 300 mA for 100 min, it was blocked in 5% skim milk for 2 h, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for all statistical analysis. Data were expressed as mean ± SD (Standard Deviation). The *t*-test was used for analyzing intergroup differences. $p<0.05$ indicated the significant difference.

Results

MF Treatment Alleviated Cerebral Edema and Neurological Deficits at Post-ICH

The concentration of Evans blue extravasation in brain tissue can directly reflect blood-brain barrier damage. As shown in Figure 1A, brain injury was more severe in ICH group and MF group relative to sham group based on the infarct size, which was the most pronounced in ICH group. By detecting cerebral edema content and neurological deficit score, it is found that blood-brain barrier destruction and cerebral edema index markedly increased in ICH group (Figure 1B, 1C). MF treatment effectively alleviated these pathological lesions. Besides, ROS content was higher in rat brain tissues of ICH group and MF group relative to controls, especially in ICH group (Figure 1D).

MF Decreased Tissue Impairment by Enhancing Antioxidant Capacity

ICH markedly impairs the antioxidant capacity of brain tissues. Here, MDA content was higher in ICH group and MF group, which was the highest in ICH group (Figure 2A). Conversely, activities of GSH, CAT and SOD decreased in ICH group relative to those in sham group. MF treatment markedly elevated these indexes (Figure 2B-2D). Western blot analyses showed that MF treatment upregulated nuclear level of Nrf2, but downregulated NF-κB expression, suggesting the activation of Nrf2 signaling pathway. Meanwhile, it also upregulated protein levels of HO-1 and NQO-1 (Figure 2E).

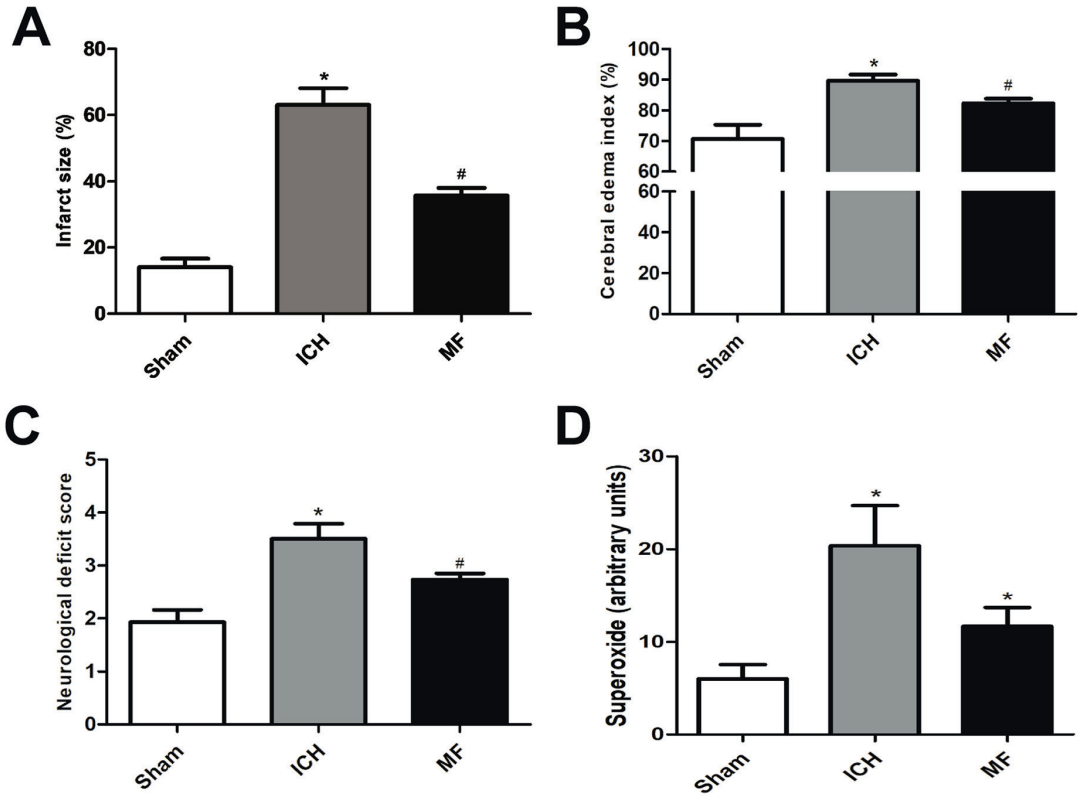

Figure 1. MF treatment alleviated neuronal edema and neurological deficits at post-ICH. ***A,*** Percent of the infarct size in rat brain tissue of each group. ***B,*** Cerebral edema index in each group. ***C,*** Neurological deficit score in each group. ***D,*** ROS production in each group. Data were presented as mean±SD, *significant difference *vs.* Sham group ($p< 0.05$); #significant difference *vs.* ICH group ($p<0.05$).

MiRNA-139 was Highly Expressed in ICH Rats

Compared with ICH group, expression level of miRNA-139 was upregulated by MF treatment, and the difference was statistically significant (Figure 3A). To explore the effects of miRNA-139 on cell viability and Nrf2 pathway, transfection efficacy of miRNA-139 mimics and inhibitor was first verified in SH-SY5Y cells by qRT-PCR (Figure 3B). SH-SY5Y cells overexpressing microRNA-139 presented higher viability, while those with microRNA-139 knockdown had lower viability than controls (Figure 3C). Furthermore, SH-SY5Y cells overexpressing microRNA-139 showed higher nuclear level of Nrf2 and lower nuclear level of NF-κB relative to those transfected with negative control. Conversely, expression patterns of Nrf2 and NF-κB were opposite in cells transfected with microRNA-139 inhibitor (Figure 3D).

MF Upregulated Nrf2 and its Downstream Gene Expressions

SH-SY5Y cells were treated with 10 mM ferrous sulfate, to establish the intracerebral hemorrhage cell model. Subsequently, to investigate the protective mechanism of MF on neurons, SH-SY5Y cells were induced with 0, 1 and 10 μM MF, respectively. High-dose MF treatment greatly upregulated nuclear level of Nrf2, but downregulated nuclear level of NF-κB (Figure 4A). Besides, microRNA-139 expression gradually upregulated with the increased concentration of MF (Figure 4B). In addition, immunofluorescence revealed that high concentration of MF markedly reduced ICH-induced ROS accumulation (Figure 4C).

Discussion

Hemorrhagic injury is mainly resulted from the hematogenous mass effect, leading to compression

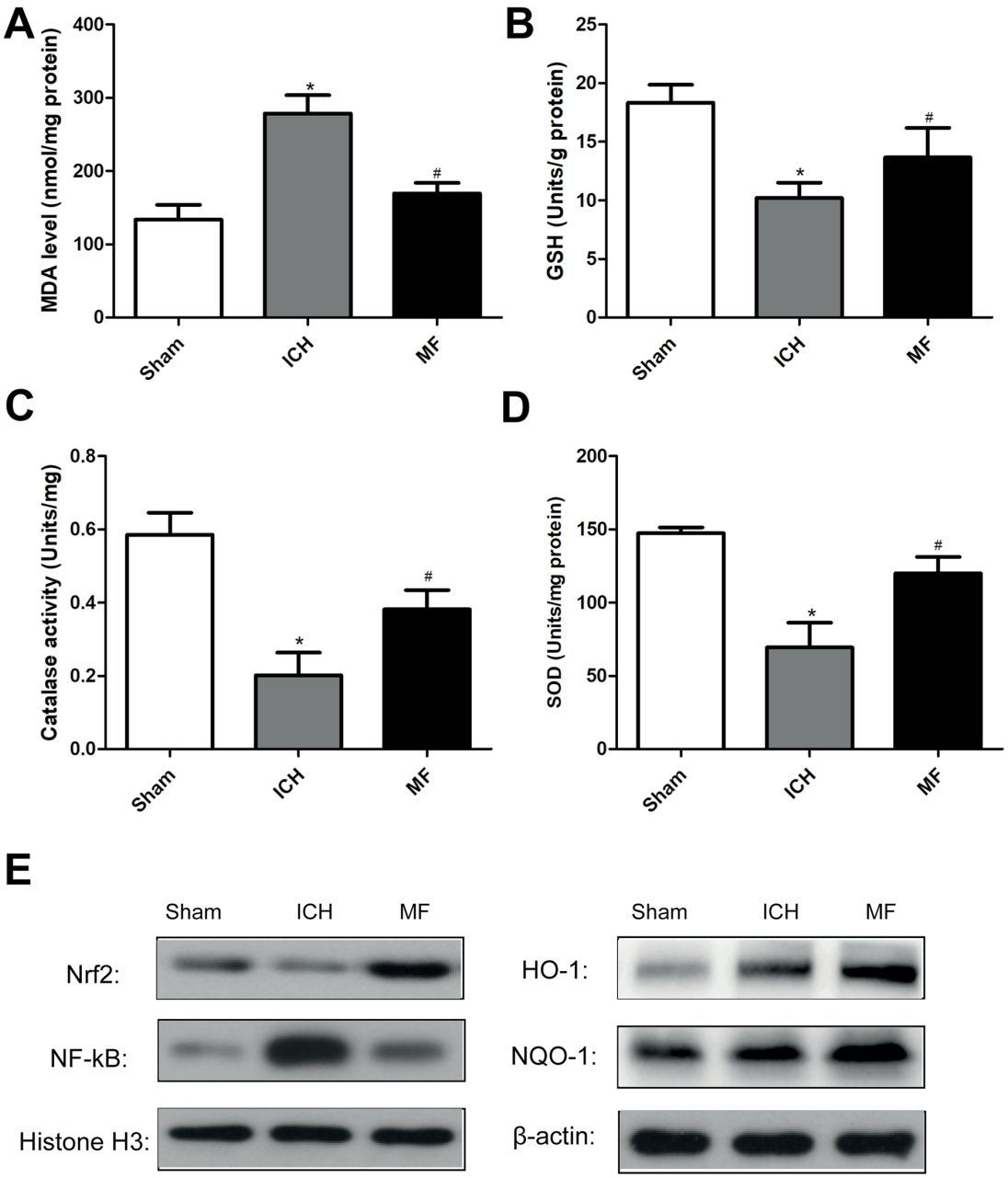

Figure 2. MF decreased tissue impairment by enhancing antioxidant capacity. ***A,*** MDA content in rat brain tissue of each group. ***B,*** GSH content in rat brain tissue of each group. ***C,*** CAT content in rat brain tissue of each group. ***D,*** SOD content in rat brain tissue of each group. ***E,*** Western blot analyses of Nrf2, NF-κB, HO-1 and NQO-1 in rat brain tissue of each group. Data were expressed as mean±SD. *significant difference *vs.* Sham group (p<0.05); #significant difference *vs.* ICH group (p<0.05).

of local microvascular bed and distal structure accompanied by micro-bleeding, midline shift, and even cerebral palsy[21,22]. However, rapid removal of hematoma does not effectively improve the neurological deficit of ICH, indicating that there are still secondary injuries at post-ICH[23]. Therefore, explorations on ICH-induced secondary injuries help to improve the clinical outcome of ICH patients. Hemorrhage-induced pathological lesions in the brain are the leading cause of brain injuries. It is considered that inflammatory response, oxidative stress, neuronal apoptosis and ischemic pathway are the major risk factors for ICH. These lesions eventually lead to cognitive dysfunction, neuronal apoptosis, brain edema and blood-brain barrier disruption. Oxidative stress particularly

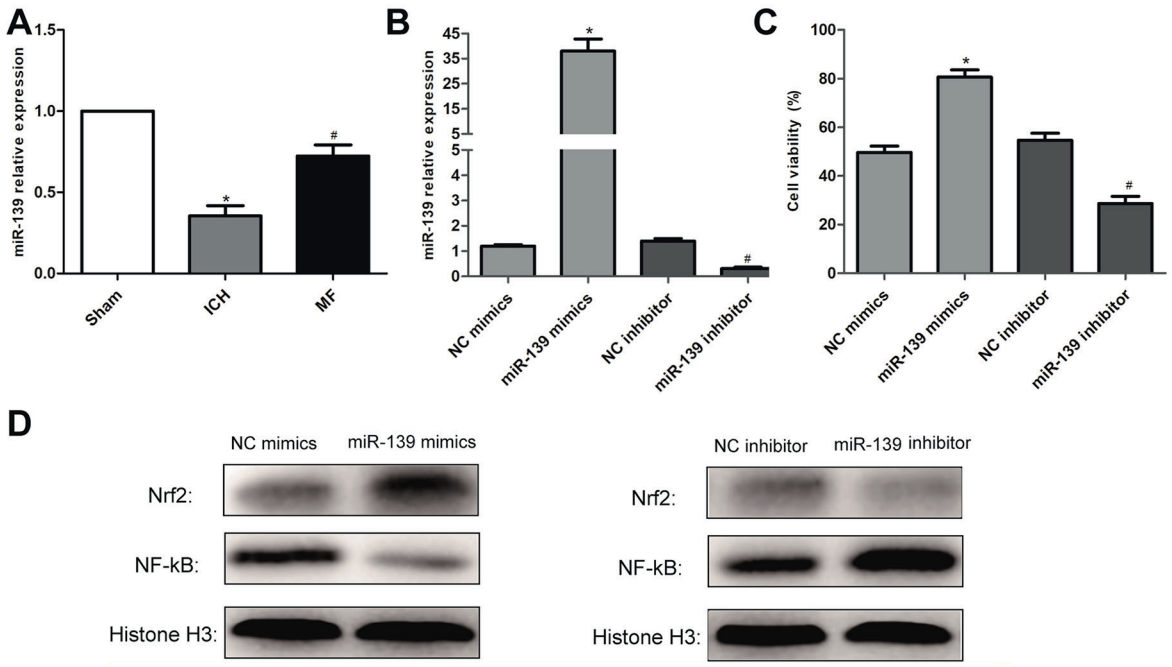

Figure 3. MiRNA-139 was highly expressed in ICH rats. ***A,*** Expression level of miR-139 in rat brain tissue of each group. ***B,*** Transfection efficacy of miRNA-139 mimics and miRNA-139 inhibitor in SH-SY5Y cells. ***C,*** =Cell viability in SH-SY5Y cells with miRNA-139 overexpression or knockdown. ***D,*** Western blot analyses of Nrf2 and NF-κB in SH-SY5Y cells with miRNA-139 overexpression or knockdown. Data were expressed as mean±SD.*significant difference *vs.* NC mimics (p<0.05); #significant difference *vs.* NC inhibitor (p<0.05).

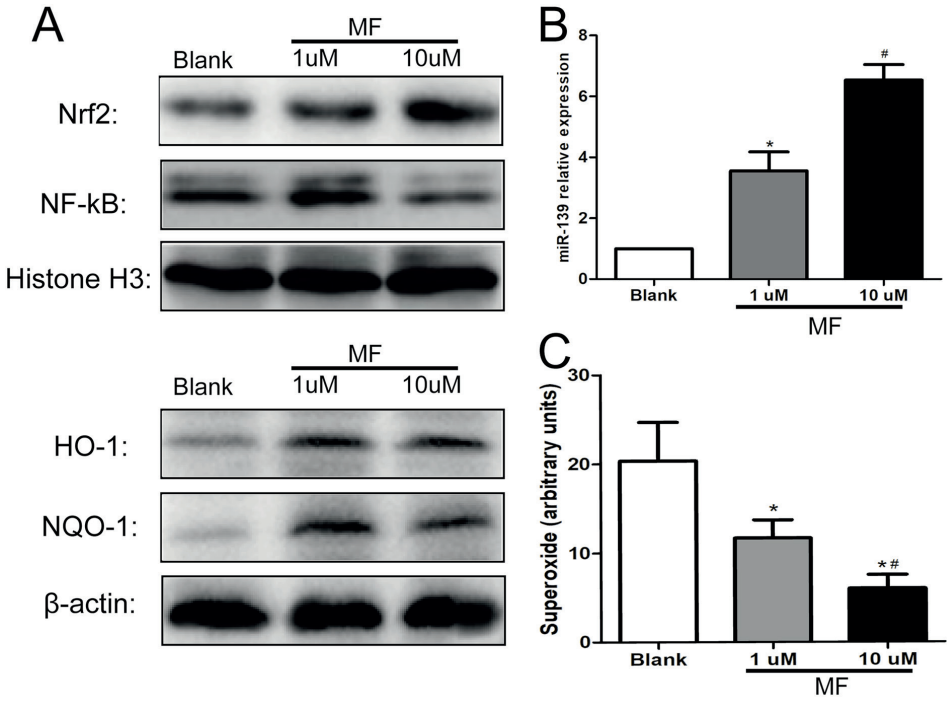

Figure 4. MiRNA-139 was highly expressed in ICH rats. ***A,*** Western blot analyses of Nrf2, NF-κB, HO-1 and NQO-1 in SH-SY5Y cells treated with 10 mM ferrous sulfate with 0, 1 and 10 μM MF. ***B,*** qRT-PCR analysis of miRNA-139 expression level in SH-SY5Y cells treated with 10 mM ferrous sulfate with 0, 1 and 10 μM MF. ***C,*** ROS production in SH-SY5Y cells treated with 10 mM ferrous sulfate with 0, 1 and 10 μM MF. Data were expressed as mean± SD. *significant difference *vs.* Blank (p<0.05); #significant difference *vs.* MF 1 μM (p<0.05).

exerts a crucial role in ICH[6-9]. Oxygen free radicals produced during metabolism attack macromolecular substances, damage functional protein synthesis, destroy cell membrane structure, and promote chemical cross-linking between proteins and nucleic acid molecules. Oxidative stress ultimately leads to cell death[24,25]. Intracellular Nrf2 is considered to be a key factor in regulating expressions of numerous antioxidants. It exerts multiple biological activities that maintain anti-inflammatory, anti-apoptotic functions, and oxidative-antioxidant balance. Nrf2-ARE pathway controls and regulates oxidative stress by regulating various antioxidant enzymes[14-17]. In recent years, microRNA-139 is found to be lowly expressed in tumor cells, which is related to tumor invasion and metastasis[24,25]. Inflammatory damage is responsible for tumor invasion and metastasis, and we thus speculated whether microRNA-139 could regulate inflammatory response[12,13]. In this study, microRNA-139 was lowly expressed in ICH rats, which was further upregulated by MF treatment. MF was previously considered as an effective radiosensitizer for hypoxic cells. Later, MF combined with three fumaric acid esters (FAE) was authorized in Germany for oral treatment of psoriasis[18, 19]. MF affects intracellular thiol levels by regulating cell redox system. It activates and stabilizes the Nrf2-ARE pathway, leading to elevated levels of reduced glutathione and inhibition of nuclear translocation of NF-κB. Therefore, a cascade of inflammatory cytokines, chemokines, and adhesion molecules in immune system cells and endothelial cells protects the pathological lesions[16,17,20]. Studies[19,20] have shown that MF has a variety of physiological functions, such as anti-inflammation, immune regulation, anti-oxidation, and promotion of the nervous system and retina development. However, the impact of MF on ICH has not been reported yet. In this paper, ICH rats presented significant histopathological changes, high oxidative stress level and low level of antioxidant capacity. MF treatment markedly alleviated ICH-induced brain injuries. The anti-oxidant effect of MF protects tissue or organ injuries[18-20]. In this experiment, expression levels of Nrf2, HO-1 and NQO-1 were markedly upregulated after MF treatment, while expression level of NF-κB was downregulated. At the cellular level, overexpression of microRNA-139 upregulated Nrf2 expression and downregulated NF-κB expression in SH-SY5Y cells as well. Moreover, high-level MF treatment significantly enhanced expressions of microRNA-139 and Nrf2, thereby protecting ICH-induced injuries. To sum up, our results verified that excessive production of ROS due to brain injuries at post-ICH resulted in NF-κB activation. MF treatment attenuated NF-κB-induced inflammatory response in brain injuries through microRNA-139/Nrf2 axis, thus protecting ICH-induced secondary injuries.

Conclusions

MF protects ICH in rats by inhibiting oxidative stress and inflammatory response through activating microRNA-139/Nrf2 axis.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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