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Original Article

Curcumin attenuates brain edema in mice with intracerebral hemorrhage through inhibition of AQP4 and AQP9 expression

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Aim: Aquaporins (AQPs) are the water-channels that play important roles in brain water homeostasis and in cerebral edema induced by brain injury. In this study we investigated the relationship between AQPs and a neuroprotective agent curcumin that was effective in the treatment of brain edema in mice with intracerebral hemorrhage (ICH).

Methods: ICH was induced in mice by autologous blood infusion. The mice immediately received curcumin (75, 150, and 300 mg/kg, ip). The Rotarod test scores, brain water content and brain expression of AQPs were measured post ICH. Cultured primary mouse astrocytes were used for *in vitro* experiments. The expression of AQP1, AQP4 and AQP9 and NF-κB p65 were detected using Western blotting or immunochemistry staining.

Results: Curcumin administration dose-dependently reduced the cerebral edema at d 3 post ICH, and significantly attenuated the neurological deficits at d 5 post ICH. Furthermore, curcumin dose-dependently decreased the gene and protein expression of AQP4 and AQP9, but not AQP1 post ICH. Treatment of the cultured astrocytes with Fe^{2+} (10–100 μmol/L) dose-dependently increased the expression and nuclear translocation of NF-κB p65 and the expression of AQP4 and AQP9, which were partly blocked by co-treatment with curcumin (20 μmol/L) or the NF-κB inhibitor PDTC (10 μmol/L).

Conclusion: Curcumin effectively attenuates brain edema in mice with ICH through inhibition of the NF-κB pathway and subsequently the expression of AQP4 and AQP9. Curcumin may serve as a potential therapeutic agent for ICH.

Keywords: curcumin; intracerebral hemorrhage; brain edema; NF-κB p65; AQP4; AQP9; astrocytes; Fe^{2+}; PDTC

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Introduction

Intracerebral hemorrhage (ICH) causes approximately 10%–20% of all strokes worldwide[1] and is associated with a high rate of fatality and morbidity[2, 3]. Spontaneous ICH accounts for 8%–20% of all strokes in high-income countries and has an annual incidence of 24–48 per 100 000[4]. Less than 50% of patients with ICH survive more than 1 year[3]. In addition to hemorrhagic volume, the development of brain edema is a major complication associated with ICH. Increasing intracranial pressure (ICP) reduces cerebral blood flow and cerebral perfusion pressure and contributes to a poor prognosis[5–7]. Current therapies for ICH, including medicinal and neurosurgical treatment, do not adequately control brain edema. There is therefore a need for new medicinal therapeutics and methods to be developed to limit post-ICH edema.

Curcumin, or turmeric, which is derived from the root of the plant *Curcuma longa* Linn, has been used as a treatment for inflammatory conditions in Ayurvedic medicine for centuries[8]. It has been reported that curcumin attenuates neurobiological deficits in animal models of different neurological disorders, including Parkinson's disease[9], brain trauma[10], ischemic stroke[11], and subarachnoid hemorrhage (SAH)[12]. The protective and therapeutic effects of curcumin are associated with its anti-inflammatory, anti-oxidative, and anti-apoptotic properties[13]. Previous studies have demonstrated that curcumin attenuates brain edema and improves neurological outcomes in ICH mice[14, 15]. However, the neuroprotective mechanisms of curcumin in ICH remain poorly understood.

Aquaporins (AQPs), a family of water-channel proteins, play an important role in water movement and homeostasis[16].

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AQP1, AQP4, and AQP9 have been extensively studied in the rodent brain, and AQP4 is the most well-studied water channel[17]. AQP1 is primarily detected in epithelial cells of the choroid plexus and has been shown to play an important role in cerebrospinal fluid formation and brain water homeostasis[18, 19]. AQP4 is the most abundant water channel in the nervous system and is mainly located on astrocytic endfeet at contacts with cerebral blood vessels. AQP9 is not only a water channel; it also facilitates the transfer of several solutes, including glycerol, urea, and monocarboxylate, suggesting that it plays an additional role in energy metabolism[17]. Many studies have shown that AQP1, AQP4 and AQP9 are associated with cerebral edema induced by several types of brain injury, including ICH, subarachnoid hemorrhage, ischemic stroke and brain trauma[10, 20–26]. However, the relationship between curcumin and AQPs has not been studied in ICH. We therefore asked whether curcumin attenuates brain edema by down-regulating AQPs after ICH.

Materials and methods

ICH mouse model

All animal experimental procedures were approved by the ethical committee of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine. C57BL/6 male mice weighing 25–30 g were randomly divided into sham (n=27), ICH (n=27), and curcumin-treated group (n=51), and maintained in a constant environment (temperature: 22±1 °C; humidity: 75%–80%) with a 12 h/12 h light/dark cycle. ICH was induced by autologous blood infusion, as has been described previously[27]. Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Mice were then placed in a stereotactic frame (RWD Life Science Co, Shenzhen, China). A 1-mm burr hole was drilled 2.3 mm lateral to the midline and 0.2 mm anterior to bregma. A volume of 30 μL of autologous blood was collected from a tail cut by Hamilton syringe. A needle was then inserted 3.5 mm into the right striatum through the burr hole. A volume of 5 μL of blood was injected into the target point at a rate of 2 μL/min with a microinfusion pump (WPI, Sarasota, FL). The injection was then paused for 7 min before the remaining portion was delivered at the same rate. When the injection was completed, the needle was left in place for 10 min to prevent possible backflow. After withdrawal of the needle, the skin was sutured. Body temperature was maintained at 37 °C throughout all of these procedures.

Drugs and chemicals

Curcumin (Purity≥98%; LKT Laboratories Inc, St Paul, MN, USA) was dissolved in DMSO and injected intraperitoneally at a dose of 75, 150, or 300 mg/kg immediately after ICH was induced. Vehicle mice received intraperitoneal injections of the same volumes of DMSO. For cultured astrocyte experiments, curcumin was dissolved in DMSO and diluted with culture medium to 20 μmol/L.

Ferrous chloride ($FeCl_2$) (Sinopharm Chemical Reagent Co, Ltd, China) was directly diluted with culture medium to the indicated concentration. Pyrrolidine dithiocarbamate (PDTC), a NF-κB inhibitor, was purchased from Beyotime (China) and used at a concentration of 10 μmol/L. All other chemicals used in this study were analytical grade.

Measurement of brain water content

Mice were sacrificed under deep pentobarbital anesthesia on day 3 (d 3) after ICH. Brain tissue was immediately weighed (wet weight) and dehydrated at 100 °C for 24 h. The samples were then reweighed to obtain a dry weight. Tissue water content was calculated with the following formula: [(wet weight-dry weight)/wet weight]×100.

Behavioral testing

The Rotarod test was used to evaluate motor coordination. Before induction of ICH, mice were placed on a rotating horizontal rod and trained first at a slow speed. After the mice were habituated, the speed was accelerated to 40 rounds/min. All mice were trained twice per day. After 3 d of training, animals that could stay on the rod for 5 min were selected for the surgical procedure. The remaining mice were not included in the study. After ICH, selected mice were tested 3 times per day on d 1, d 2, d 3, d 5, d 7, and d 14, and the average latency to falling was documented.

Astroglial cell culture

Primary cultures of astrocytes were prepared from d 1–3 newborn C57BL/6 mouse brains. After careful removal of the meninges under a dissecting microscope, cerebral tissues were homogenized mechanically and digested in 0.25% trypsin for 10 min. Isolated cells were suspended in Dulbecco's modified Eagle's medium (DMEM) in 10% (v/v) fetal bovine serum (FBS) and plated onto 10-cm dishes at a density of 10×10^6 cells. Cells were cultured at 37 °C in an incubator with a 5% CO_2 air-humidified atmosphere. The culture medium was renewed after 24 h, and then every 2 d thereafter.

Relative quantitative real-time polymerase chain reaction (PCR) analysis

Total RNA was isolated 24 h after ICH from brain blocks, including hematoma and perihematomal tissues, using Trizol reagent (Invitrogen, USA). Real-time PCR was performed using a One Step SYBR® PrimeScript™ PLUS RT-PCR Kit (Takara Bio Inc, China). The primers used to amplify the target genes were as follows: AQP1: 5′-TGCGTTCTGGCCACCACTGAC-3′ (forward), 5′-GATGTCGTCAGCATCCAGGTC-3′ (reverse); AQP4: 5′-CTGGAGCCAGCATGAATCCAG-3′ (forward), 5′-TTCTTCTCTTCTCCACGGTCA-3′ (reverse); AQP9: 5′-CCTTCTGAGAAGGACCGAGCC-3′ (forward), 5′-CTTGAACCACTCCATCCTTCC-3′ (reverse); GADPH: 5′-AGGTCGGTGTGAACGGATTTG-3′ (forward), 5′-TGTAGACCATGTAGTTGAGGTCA-3′ (reverse). All primers were constructed by Invitrogen Corp (Shanghai). Relative levels of mRNA expression were calculated using SDS software (Applied Biosystems, HT7900, USA).

Immunohistological staining

After being deeply anesthetized, mice were perfused with saline followed by fixation with 4% paraformaldehyde (PFA) in 0.1 mol/L phosphate buffer (PBS). Brains were dehydrated in 20%–30% sucrose after an overnight post-fixation in 4% PFA. Brain sections (20 μm) were washed, blocked in 10% bovine serum albumin (BSA) for 30 min, and incubated overnight at 4 °C with primary antibodies: rabbit anti-AQP4 (1:200, sc-20812, Santa Cruz Biotechnology, USA), rabbit anti-AQP9 (1:100, sc-28623, Santa Cruz Biotechnology, USA), and mouse anti-glial fibrillary acidic protein (GFAP) (1:200, MAB360, Millipore, USA). Sections were then washed and incubated with secondary antibodies: Alexa Fluor 488 donkey anti-rabbit IgG (1:500, A21206, Invitrogen, USA) and Alexa Fluor 594 donkey anti-mouse IgG (1:500, A21203, Invitrogen, USA) for 1 h. Microvessels were detected by Texas Red-labeled Lycopersicon Esculentum Lectin (1:200, TL-1176, Vector Laboratories, USA) incubated with primary antibody. A Leica confocal laser-scanning microscope (Leica, Wetzlar, Hesse, Germany) was used to acquire confocal microscopic images.

Immunocytochemistry

Astrocytes were plated onto glass slides. Following treatment with Fe^{2+} or curcumin, cells were fixed in 4% PFA for 15 min and washed with 0.1 mol/L PBS. After treatment with 0.3% Triton, cells were blocked in 10% BSA for 30 min, and then incubated with primary antibodies overnight at 4 °C. The concentrations of antibodies and other steps for immunocytochemistry were the same as those used for immunohistological staining.

Western blotting

Astrocytes and brain samples (perihematomal tissues at 72 h) were collected in lysis buffer. A nuclear and cytoplasmic protein extraction kit (P0027, Beyotime, China) was used to separate nuclear and cytoplasmic proteins. Protein concentrations were quantified using a bicinchoninic acid protein assay kit (Pierce Biotechnology, USA). Then, 50 μg of protein was loaded onto a 10% polyacrylamide gel for electrophoresis, and then transferred onto a polyvinylidenedifluoride membrane. Blots were blocked with 5% non-fat milk. After being washed, blots were incubated overnight at 4 °C in primary antibody: AQP4 (1:500, sc-20812, Santa Cruz Biotechnology, USA), AQP9 (1:500, sc-28623, Santa Cruz Biotechnology, USA), NF-κB p65 (1:500, sc-372, Santa Cruz Biotechnology, USA), β-actin (1:1000, 8457, Cell Signaling Technology, USA), β-tubulin (1:1000, 2128, Cell Signaling Technology, USA), or Histone H3 (1:1000, 4499, Cell Signaling Technology, USA), followed by 1-h incubation with secondary antibody: Goat anti-rabbit HRP (1:2000, HA-1001-100, HangZhouHuaAn Biotechnology Co, China). Blots were then reacted with SuperSignal West Pico Substrate (ThermoFisher Scientific, USA). Chemiluminescence was detected using an imaging system and quantified using Quantity One software (Bio-Rad, USA).

Statistical analysis

All results were expressed as the mean±SD and analyzed with GraphPad Prism (version 5.01, GraphPad Software, Inc) using one-way analysis of variance followed by a Student-Newman-Keuls test, or a two-way analysis of variance followed by Bonferroni post hoc test. A *P*-value of <0.05 was considered statistically significant.

Results

Curcumin reduced cerebral edema and attenuated neurological deficits

Brain water content was significantly increased on d 3 after ICH in the ipsilateral hemisphere (83.9%±0.6% after ICH *vs* 78.1%±0.5% in sham, $P<0.001$). The increase in brain edema following ICH was attenuated in a dose-dependent manner by a single injection of curcumin (75, 150, and 300 mg/kg) administered immediately after ICH induction (81.0%±0.8%, 79.9%±0.2%, 79.5%±0.6%, respectively; $P<0.001$ *vs* ICH). Brain water content was not significantly different between any of the treatment groups in the contralateral hemisphere (78.7%±0.6%, 78.5%±0.6%, 78.17%±0.5% *vs* 79.2%±0.2% in ICH; $P>0.05$) (Figure 1A).

A significant reduction in neurological behavior after ICH was detected in all mice by the Rotarod test (Figure 1B). After treatment with curcumin (150 mg/kg), behavioral outcomes were improved significantly on d 5 (248.2±39.6 s *vs* 174.3±34.2 s, $P<0.05$), d 7 (231±40.5 s *vs* 165.4±45.2 s, $P<0.05$), and d 14 (274.9±35.5 s *vs* 172.4±26.7 s, $P<0.05$) after ICH compared to the ICH group.

Curcumin reduced gene expression of AQP4 and AQP9, not AQP1

As shown in Figure 2A, AQP1, AQP4, and AQP9 mRNA were increased significantly 24 h post ICH (6.1±0.2, 9.1±0.3, 6.9±0.5-fold increase; $P<0.001$ *vs* sham). Curcumin (150 mg/kg) administration reduced gene expression of AQP4 (2.7±0.1 *vs* 9.1±0.3-fold increase in ICH, $P<0.001$) and AQP9 (4.0±0.4 *vs* 6.9±0.5-fold increase in ICH, $P<0.001$), but not AQP1 (5.9±0.1 *vs* 6.1±0.2-fold increase in ICH, $P>0.05$). In contrast, a single injection of curcumin (75, 150, and 300 mg/kg) decreased AQP4 (8.1±1.0, 4.1±0.2, 3.9±0.3 *vs* 10.1±1.1, $P<0.01$) and AQP9 (6.1±0.6 *vs* 7.3±0.8, $P>0.05$; 4.0±0.4, 3.5±0.7 *vs* 7.3±0.8, $P<0.01$) gene expression, in a dose-dependent manner, at 24 h after hemorrhage (Figure 2B).

Curcumin inhibited protein expression of AQP4 and AQP9

Immunofluorescence staining for AQP4 on d 3 after ICH showed that AQP4 expression was significantly elevated in the perihematomal area and that AQP4 was increased in perivascular astrocyte endfeet (Figure 3Aa and 3Ab). Curcumin (150 mg/kg) decreased AQP4 expression compared to that in the vehicle-treated ICH group. Western blot analysis confirmed that AQP4 protein expression was elevated in the perihematomal area at 72 h (1.05±0.02 *vs* 0.46±0.01 in sham, $P<0.001$) following ICH. Curcumin injection (75, 150, and 300 mg/kg) attenuated this post-ICH induction of AQP4 (0.90±0.03, 0.56±0.01, 0.50±0.03 *vs* 1.05±0.02 in the vehicle-treated ICH

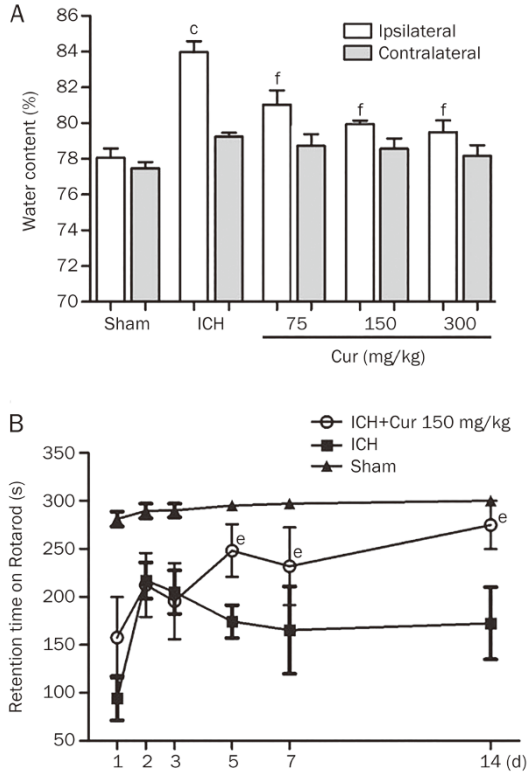

Figure 1. Curcumin reduced cerebral edema and attenuated neurological deficits. (A) Curcumin reduced brain edema in a dose-dependent manner at d 3. Curcumin (Cur; 75, 150, or 300 mg/kg) was administered to mice (n=6/group) immediately after ICH. Significant differences were observed between groups in the ipsilateral hemisphere (^{c}P<0.01 *vs* Sham, ^{f}P<0.01 *vs* ICH), no significant differences were found in the contralateral hemisphere. Values are given as the mean±SD. (B) Neurological deficits were attenuated on the 5th day after ICH, compared to the vehicle-treated ICH group (^{e}P<0.05). On the 14th day, results of Rotarod test were near the level of the sham group. Values are given as the mean±SD from 9 mice/group.

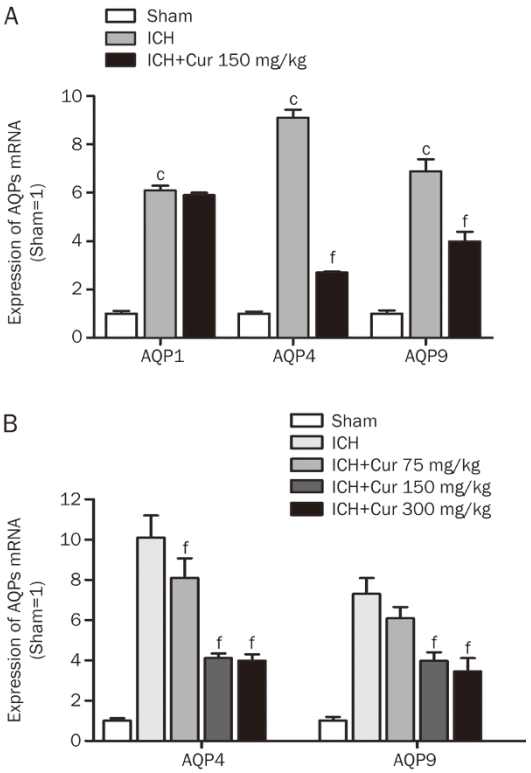

Figure 2. Curcumin reduced the gene transcription of AQP4 and AQP9, but not AQP1. (A) AQP1, AQP4 and AQP9 mRNA levels were increased significantly at 24 h after ICH (^{c}P<0.01 *vs* Sham). AQP4 and AQP9 mRNA levels were markedly decreased by curcumin (150 mg/kg) (^{f}P<0.01 *vs* ICH), but no significant difference was found in AQP1 (P>0.05). Values are given as the mean±SD from 3 mice/group. (B) AQP4 and AQP9 mRNA levels were reduced by curcumin in a dose-dependent manner (^{f}P<0.01 *vs* ICH). Values are given as the mean±SD from 3 mice/group.

group, P<0.001) (Figure 3Ac and 3Ad).

A similar effect was observed in AQP9 expression, in that immunofluorescence showed that AQP9 expression was markedly increased on astrocytes in the perihematomal area and that this effect was inhibited by curcumin administration (150 mg/kg) (Figure 3Ba). Consistent with results obtained by Western blot, the intensity of AQP9 expression was strongly increased after hemorrhage (0.91±0.01 *vs* 0.44±0.01 in sham, P<0.001). This effect was decreased by curcumin (75, 150, and 300 mg/kg) in a dose-dependent manner (0.89±0.01 *vs* 0.91±0.01 in ICH group, P>0.05; 0.69±0.04, 0.39±0.04 *vs* 0.91±0.01 in ICH group, P<0.001) (Figure 3Bb and 3Bc).

Fe^{2+} increased protein expression of AQP4 and AQP9 in astrocytes

In an *in vitro* assay, immunofluorescence staining showed that the protein levels of both AQP4 (Figure 6Aa) and AQP9 (Figure 6Ba) were increased in astrocytes at 12 h after treatment with Fe^{2+} (50 μmol/L). Western blot analysis demonstrated that as the concentration of Fe^{2+} (10, 25, 50, and 100 μmol/L) increased, the levels of AQP4 (0.74±0.03, 0.85±0.04, 1.13±0.02, 1.02±0.03 *vs* 0.44±0.04 in control, P<0.001) and AQP9 (0.41±0.04 *vs* 0.39±0.06 in control, P>0.05; 0.88±0.03, 1.00±0.05 *vs* 0.39±0.06 in control, P<0.001) were elevated, reaching a peak at 50 μmol/L (Figure 4).

Curcumin inhibited the activation of the NF-κB pathway in astrocytes

NF-κB p65 protein expression was increased in astrocytes after treated by Fe^{2+}. As shown in Figure 5A, the level of p65 expression (0.70±0.02 *vs* 0.52±0.13 in control, P>0.05; 0.92±0.02, 1.33±0.03, 0.87±0.02 *vs* 0.52±0.13 in control, P<0.001) was elevated in a manner following the concentration of Fe^{2+} (10, 25, 50, and 100 μmol/L), reaching a peak at 50 μmol/L. After treatment with 50 μmol/L of Fe^{2+}, expression of p65 was increased in cytoplasm (1.12±0.07 *vs* 0.73±0.03 in control, P<0.01), and nuclear translocation of p65 was activated (1.00±0.05 *vs* 0.61±0.13 in control, P<0.01). Co-treatment with curcumin and Fe^{2+} reduced expression of p65 in cytoplasm (0.95±0.03 *vs* 1.12±0.07 in Fe^{2+}-only treated group, P<0.05), and nuclear translocation was also inhibited (0.62±0.11 *vs* 1.00±0.05

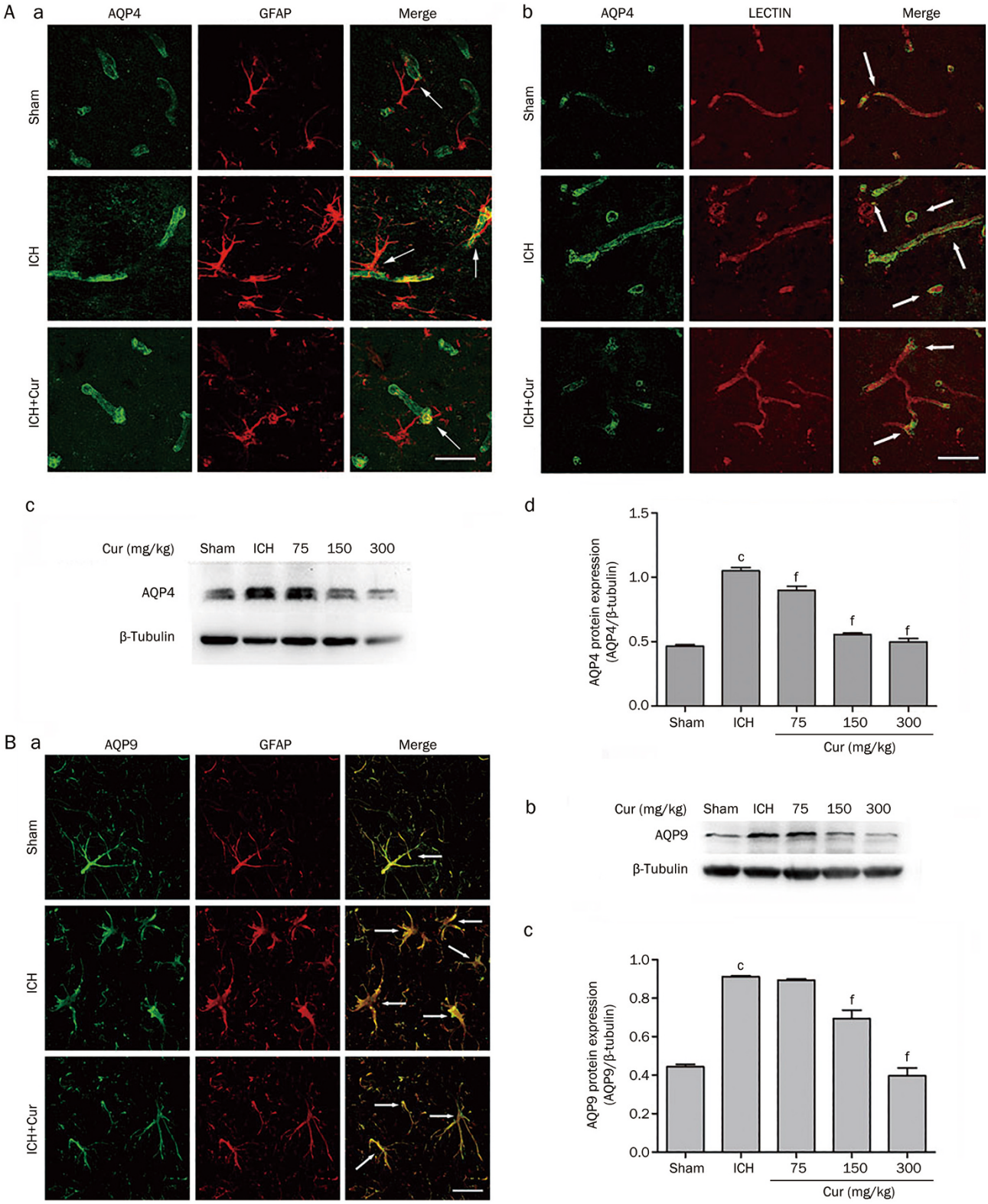

Figure 3. Curcumin inhibited the protein expression of AQP4 and AQP9 in ICH mice. (A) AQP4 protein expression was increased significantly in the perihematomal area over 3 d after ICH, and especially in perivascular astrocyte endfeet (white arrow). Curcumin treatment (150 mg/kg) decreased AQP4 expression. The results of Western blot analysis showed that AQP4 expression was strongly induced on d 3 after ICH. Curcumin treatment inhibited AQP4 expression in a dose-dependent manner. (B) Immunoreactivity of AQP9 in astrocytes was increased significantly over 3 d after ICH (white arrow). Curcumin treatment (150 mg/kg) reduced AQP9 expression compared to that in the ICH group. AQP9 protein quantitation shown that AQP9 protein expression was markedly increased on d 3 after ICH, and down-regulated by curcumin in a dose-dependent manner. Scale bar=10 μm. Values are mean±SD from 3 mice/group. $^{c}P<0.01$ *vs* Sham, $^{f}P<0.01$ *vs* ICH.

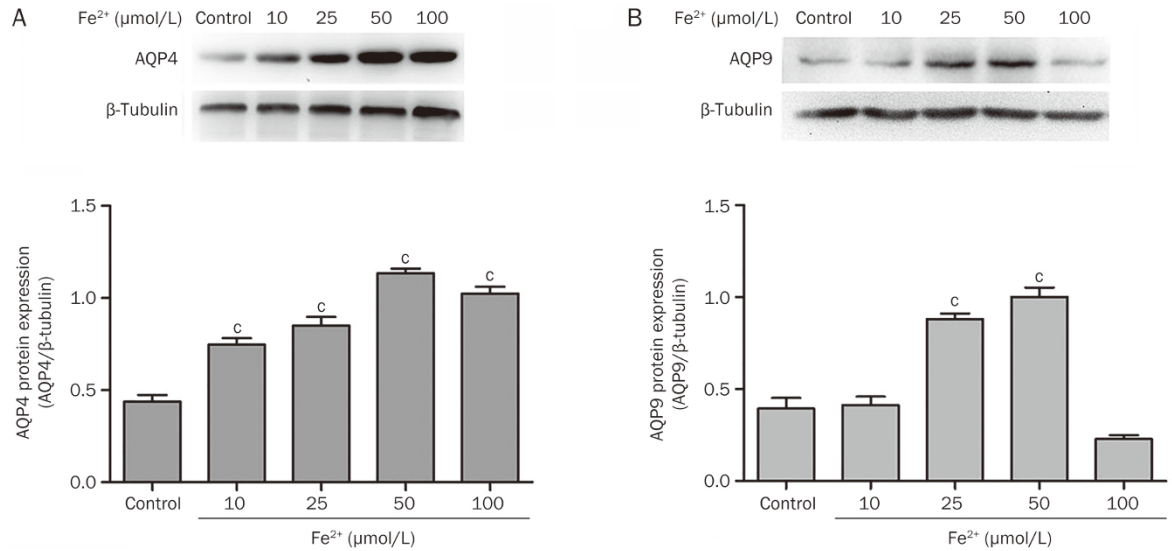

Figure 4. Fe^{2+} increased the protein expression of AQP4 and AQP9 in astrocytes. Representative Western blot at 12 h after treatment with Fe^{2+} showed that AQP4 (A) and AQP9 (B) protein expression were up-regulated as the concentration of Fe^{2+} increased, reaching a peak at 50 μmol/L. Values are mean±SD from three independent experiments. $^{c}P<0.01$ *vs* control.

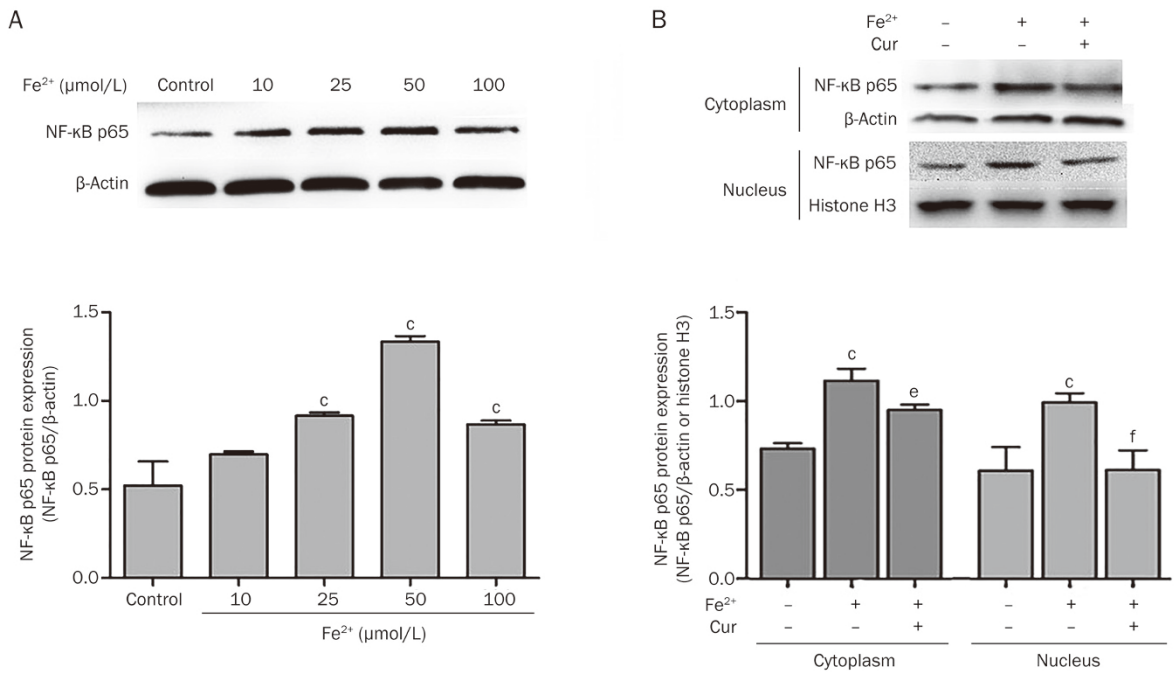

Figure 5. Curcumin inhibited the activation of the NF-κB pathway in astrocytes. (A) Total p65 protein expression was increased 12 h after treatment with Fe^{2+}, compared to controls, reaching a peak at 50 μmol/L. (B) NF-κB p65 expression in the cytoplasm and nucleus were quantitated, respectively, at 12 h after treatment with Fe^{2+}. After treatment with Fe^{2+} (50 μmol/L), p65 in the cytoplasm and nucleus were elevated. Following co-treatment with Fe^{2+} and curcumin (20 μmol/L), p65 expression in the cytoplasm and nucleus was inhibited. Values are mean±SD from three independent experiments. $^{c}P<0.01$ *vs* control; $^{e}P<0.05$, $^{f}P<0.01$ *vs* Fe^{2+}.

in Fe^{2+}-only treatment group, $P<0.01$) (Figure 5B).

Curcumin down-regulated AQP4 and AQP9 expression via the NF-κB pathway

As mentioned previously, Fe^{2+} increased AQP4 and AQP9 protein expression in astrocytes. Co-treatment with Fe^{2+} and curcumin (20 μmol/L) or PDTC (10 μmol/L), a NF-κB inhibitor, reduced Fe^{2+} induced AQP4 expression, analyzed by immunofluorescence staining. Western blot analysis resulted the same outcomes: Fe^{2+} and curcumin, 0.84±0.06, *vs*

1.06±0.05 in the Fe^{2+}-only treatment group, $P<0.01$; Fe^{2+} and PDTC, 0.79±0.05, *vs* 1.06±0.05 in the Fe^{2+}-only treatment group, $P<0.001$ (Figure 6Ab). Similar outcomes were found for AQP9 (Figure 6Bb); AQP9 protein expression was inhibited by curcumin and PDTC: curcumin, 0.89±0.04, *vs* 1.19±0.04 in the Fe^{2+}-only treatment group, $P<0.001$; PDTC, 0.73±0.06, *vs* 1.19±0.04 in the Fe^{2+}-only treatment group, $P<0.001$.

Discussion

The present study demonstrated that curcumin, a natural polyphenolic compound, attenuated ICH induced cerebral edema and neurological deficits. This effect was associated

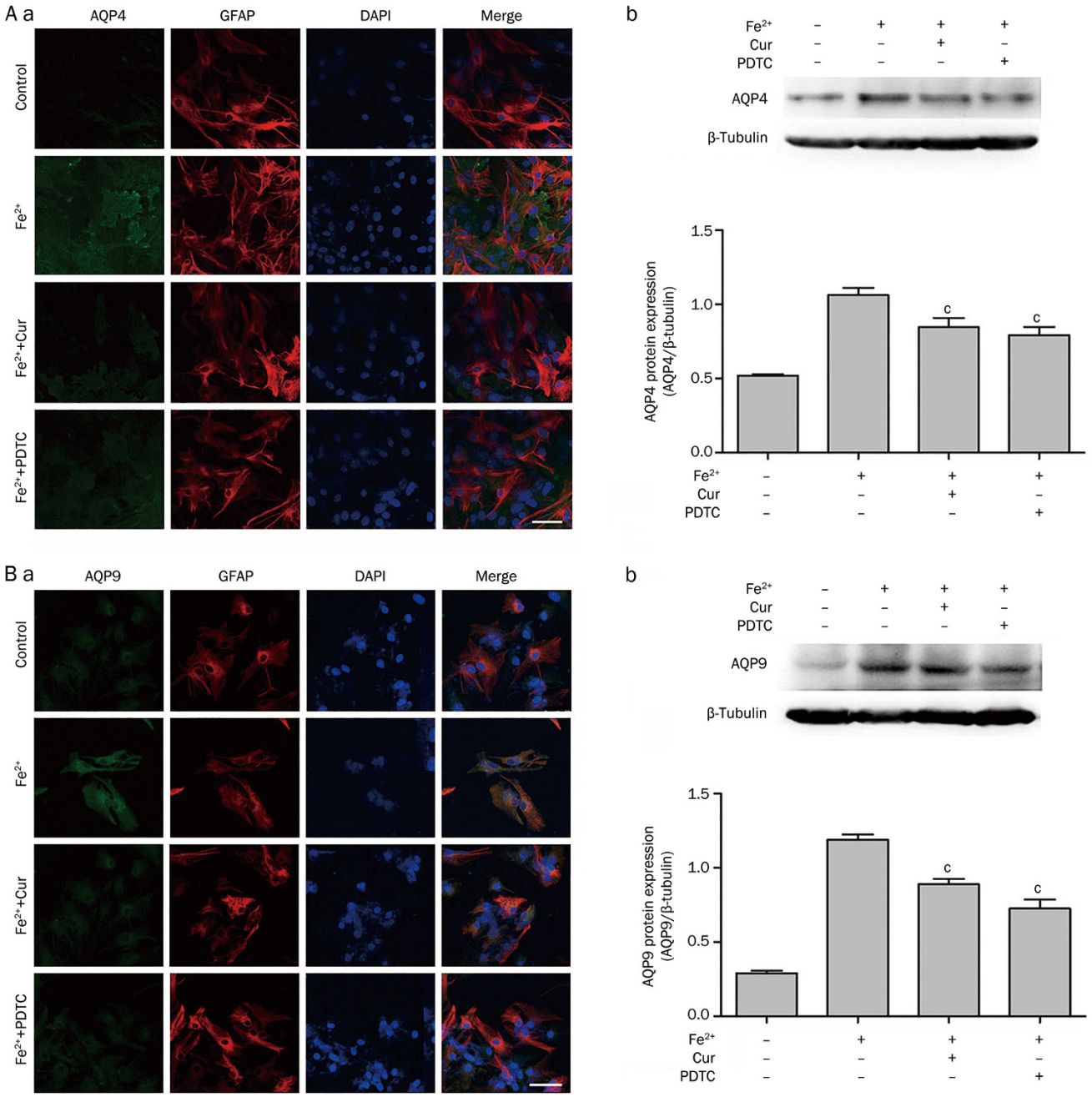

Figure 6. Curcumin or PDTC treatment inhibited AQP4 and AQP9 expression induced by Fe^{2+}. (A) Immunofluoresence was performed at 12 h after treatment with Fe^{2+} (50 μmol/L), and fluorescence intensity of AQP4 was enhanced. After co-treatment with Fe^{2+} and curcumin (20 μmol/L) or Fe^{2+} and PDTC (10 μmol/L), AQP4 expression was decreased. The Western blot analysis showed that AQP4 expression was increased at 12 h after treatment with Fe^{2+} (50 μmol/L) and inhibited by co-treatment with Fe^{2+} and curcumin (20 μmol/L) or Fe^{2+} and PDTC (10 μmol/L). (B) The fluorescence intensity of AQP9 was increased at 12 h after treatment with Fe^{2+} (50 μmol/L) and inhibited by co-treatment with Fe^{2+} and curcumin (20 μmol/L) or Fe^{2+} and PDTC (10 μmol/L). AQP9 protein quantitation proved that AQP9 expression was up-regulated at 12 h after treatment with Fe^{2+} (50 μmol/L) and that this increase was reversed by co-treatment with Fe^{2+} and curcumin (20 μmol/L) or Fe^{2+} and PDTC (10 μmol/L). Scale bar=10 μm. Values are mean±SD from three independent experiments. $^{c}P<0.01$ *vs* Fe^{2+}.

with reduced activation of the NF-κB pathway, which was activated by Fe^{2+}. It was also associated with the degradation of hemoglobin and decreased expression of AQP4 and AQP9 in astrocytes.

Brain edema and related increases in ICP following ICH contribute to herniation-related deaths and neurological deficits. A higher degree of edema is directly proportional to a poorer prognosis in patients. Perihematomal edema develops immediately after ICH, peaking around the third or fourth day in experimental ICH models, and between d 10–20 in humans[28]. In our study, d 3 was selected as a time-point for the water content tests. We found that curcumin reduced cerebral edema and improved performance on neurobehavioral tests following ICH. We noted that, in Rotarod tests, curcumin normalized ICH models to almost sham levels over the 2 weeks following ICH. Together with other reports[14, 15], these results suggest that curcumin attenuates the damage caused by intracerebral hemorrhage by inhibiting matrix metalloproteinase-mediated injury to the blood-brain barrier and cerebral edema. Curcumin therefore has therapeutic potential for treating ICH.

AQP1, AQP4, and AQP9 have been shown to play critical roles in edema formation in different types of brain diseases[17]. AQP1 expression has been reported to be elevated in human astrocytes after SAH[26] and in cortical endothelial cells in rat SAH models[29], suggesting an important role in edema formation. Nesic *et al* reported[30] that AQP1 expression was induced in a model of long-term rodent spinal injury, while astrocytic AQP1 expression contributed to the secretion of cerebrospinal fluid during cyst formation and the migration of astrocytes. Neuronal AQP1 expression therefore suggested a role in structural plasticity and neuronal repair. All of these results illustrate the complex pathological roles of AQP1. One study[30] also found that hypertonicity affected AQP4, but not AQP1 expression, suggesting that AQP1 and AQP4 expression is regulated by different mechanisms. Several studies have shown that acute brain injuries, including ICH, ischemic stroke, and brain trauma, alter AQP4 and AQP9 expression, and that decreased expression or gene knockout of AQP4 and AQP9 ameliorates brain edema and neurological deficits[10, 20–25, 31]. The level and duration of AQP4 expression was temporally correlated with the degree of brain edema in a middle cerebral artery (MCA) occlusion model. AQP4 expression has also been observed at 48 h in transient occlusions. In a mild stroke model (30 min of MCA occlusion), up-regulated AQP4 expression was detected for up to 7 d, whereas in a severe stroke model, elevated AQP4 expression persisted for up to 28 d[32, 33]. The pattern of AQP4 expression in cerebrovascular diseases has revealed that this molecule plays a critical role in regulating water movement during brain edema[17]. AQP9 expression was increased in astrocytes after ischemic injury, but AQP9 up-regulation did not correlate with the degree of brain edema as has been documented in AQP4 studies, revealing that AQP9 does not play a significant role in brain edema after cerebral ischemia. AQP9 does, however, facilitate lactate movement between astrocytes and neurons, suggesting that it plays complex roles in energy metabolism and neurorepair[25, 32]. In the present study, AQP1, AQP4, and AQP9 gene expression were increased after ICH. AQP4 and AQP9, but not AQP1, were decreased after treatment with curcumin. These novel findings suggest that curcumin could be used as an AQP4 and AQP9 inhibitor in the clinical treatment of ICH patients.

Iron, a byproduct of hemoglobin degradation, has been shown to play a major role in ICH-induced brain edema and brain damage[34–37]. Iron overload caused by ICH is an important contributor to neurobiological deficits, brain edema, inflammation, neuron apoptosis and brain atrophy. These effects can be reduced with deferoxamine, an iron chelator[38–45]. However, two studies[46, 47] have reported that deferoxamine reduces the level of iron, but does not improve outcomes, in a collagenase-induced ICH model. In our experimental design, Fe^{2+} was added to cultured astrocytes and AQP4 and AQP9 protein expression were strongly induced, suggesting that iron was related to cytotoxic edema. This phenomenon was inhibited by co-treatment of Fe^{2+} with curcumin. These results, in combination with those of Min-chao DAI in 2013[48], showed that curcumin reduced iron-induced neurotoxicity in primary cortical neurons by attenuating necroptosis and that neuroprotection by curcumin affected not only neurons but also astrocytes.

Previous studies have demonstrated that oxidative stress and inflammation are induced by ICH and the degradation of blood cells, and especially by thrombin and iron[28, 49]. Tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) are increased after ICH or treatments inducing the degradation of blood cells. The transcription factor NF-κB, which regulates IL-1β and TNF-α, is also immediately activated following ICH[49]. Consistent with these findings, our results show that NF-κB and p65 subunit expression increases as the concentration of Fe^{2+} increases. In 1995, Singh *et al* showed that curcumin is a potent inhibitor of NF-κB and that it acts by down-regulating p65 nuclear translocation and AP-1 binding factors[50]. Recent studies have also shown that curcumin is an NF-κB inhibitor in different diseases, including brain trauma[10] and oxygen-glucose deprivation[11]. As shown in Figure 5, p65 expression in the cytoplasm and nucleus were both down-regulated by curcumin. Co-treatment with Fe^{2+} and curcumin or PDTC revealed that AQP4 and AQP9 are decreased in co-treated astrocytes compared to those treated with Fe^{2+} only. We conclude that curcumin reduced AQP4 and AQP9 expression by inhibiting the NF-κB pathway.

In 2001, Cheng *et al*[51] reported that oral administration of up to 8 g/d of curcumin resulted in safe bioactivity; serum concentration peaked at 1–2 h and gradually declined over 12 h. Following on these results, which support the use of curcumin as a strong anti-inflammation and anti-oxidative stress agent, curcumin was tested in several clinical trials over a decade. Positive effects were observed in patients with pre-malignant lesions[51], colorectal cancer[52], Alzheimer's disease[53], colorectal neoplasia[54], chronic periodontitis[55], radiation dermatitis[56], mastitis[57], aphthous stomatitis[58] and osteoarthritis[59], but not in patients with psoriasis vulgaris[60]. Unfortunately, at the present time, there are no reports of

clinical trials aimed at studying the effect of curcumin on ICH. Well-designed clinical trials are needed to study and verify the neuroprotective effect of curcumin in ICH patients.

Conclusions

The results of this study suggest that curcumin attenuates ICH-induced brain edema by inhibiting AQP4 and AQP9, but not AQP1, and that it acts through the NF-κB signaling pathway. Curcumin is an effective anti-inflammatory agent and may therefore have therapeutic potential for the treatment of ICH.

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Author contribution

Liu-guan BIAN, Bao-feng WANG, and Zhen-wen CUI participated in the study design and manuscript preparation; Bao-feng WANG, Zhen-wen CUI, Zhi-hong ZHONG, and Yu-hao SUN performed the experiments; Bao-feng WANG, Zhen-wen CUI, Qing-fang SUN, and Guo-yuan YANG analyzed data; Liu-guan BIAN was responsible for supervision and funding. All authors read and approved the final version of the manuscript.

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