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Curcumin plays neuroprotective roles against traumatic brain injury partly via Nrf2 signaling

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ABSTRACT

Traumatic brain injury (TBI), which leads to high mortality and morbidity, is a prominent public health problem worldwide with no effective treatment. Curcumin has been shown to be beneficial for neuroprotection in vivo and in vitro, but the underlying mechanism remains unclear. This study determined whether the neuroprotective role of curcumin in mouse TBI is dependent on the NF-E2-related factor (Nrf2) pathway. The Feeney weight-drop contusion model was used to mimic TBI. Curcumin was administered intraperitoneally 15 min after TBI induction, and brains were collected at 24 h after TBI. The levels of Nrf2 and its downstream genes (*Hmox-1*, *Nqo1*, *Gclm*, and *Gclc*) were detected by Western blot and qRT-PCR at 24 h after TBI. In addition, edema, oxidative damage, cell apoptosis and inflammatory reactions were evaluated in wild type (*WT*) and *Nrf2*-knockout (Nrf2-KO) mice to explore the role of Nrf2 signaling after curcumin treatment. In wild type mice, curcumin treatment resulted in reduced ipsilateral cortex injury, neutrophil infiltration, and microglia activation, improving neuron survival against TBI-induced apoptosis and degeneration. These effects were accompanied by increased expression and nuclear translocation of Nrf2, and enhanced expression of antioxidant enzymes. However, Nrf2 deletion attenuated the neuroprotective effects of curcumin in *Nrf2*-KO mice after TBI. These findings demonstrated that curcumin effects on TBI are associated with the activation the Nrf2 pathway, providing novel insights into the neuroprotective role of Nrf2 and the potential therapeutic use of curcumin for TBI.

1. Introduction

Traumatic brain injury (TBI) is defined as a brain damage resulting from an external mechanical force, and contributes to a substantial number of deaths and disability, with many victims having functional impairments such as motor and sensory dysfunctions, and even cognitive deficits (Maas et al. 2008; Jennekens et al. 2010). An estimated 10 million individuals worldwide are diagnosed with TBI yearly (Corrigan et al. 2010; Bang et al. 2012). Both primary and secondary brain injuries compose the complex disorder caused by TBI. Tissue loss and cell death cause the primary injury. The inflammatory response from activated microglia as well as recruited neutrophils and macrophages, and reactive oxygen species (ROS) from blood metabolites contribute to secondary brain damage and subsequently cause a progressive damage (Ziebell and Morganti-Kossmann 2010; Helmy et al. 2011). Oxidative stress and inflammatory reaction are recognized as the important mechanisms of TBI-caused secondary injury (Cornelius et al. 2013; Tumer et al. 2013). Theoretically, improving antioxidant response and reducing inflammation would alleviate TBI-induced secondary brain damage (Cheng et al. 2016).

Nuclear factor erythroid 2 related factor 2 (Nrf2), a basic leucine zipper (bZIP) protein, is largely considered a key regulator of endogenous defense against oxidative stress in the brain. Under physiological conditions, Nrf2 is mainly located in the cytoplasm. In response to oxidative stress, Nrf2 translocates into the nucleus and heterodimerizes with the small Maf or Jun proteins, followed by binding to specific DNA sites termed antioxidant response elements (ARE) or electrophile response elements (EpRE) to initiate the transcription of cytoprotective genes in the nucleus (Itoh et al. 1997; Venugopal and Jaiswal 1998). Emerging evidence indicates that Nrf2 plays an important protective role in brain injury and neurodegenerative diseases, as deletion of Nrf2 exacerbates TBI-induced acute oxidative damage and subsequent neurological deficits in mice (Lu et al. 2015); meanwhile, insufficient Nrf2 activation in humans is linked to chronic

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neurodegenerative diseases (Sandberg et al. 2014). In addition, Nrf2 activation ameliorates TBI induced damage, similar to sulforaphane (Zhao et al. 2007), melatonin (Ding et al. 2014), carnosic acid (Miller et al. 2015), and tert-butyl hydroquinone (Lu et al. 2014), HPC, suggesting that modulating the transcription factor Nrf2 is a potential strategy to treat TBI-induced oxidative stress and inflammatory response.

Curcumin, a phytochemical compound extracted from *Curcuma longa* rhizomes, has been extensively studied for its multiple biological activities, including anti-inflammatory, antioxidant, anti-infective properties (Agarwal et al. 2011). Curcumin has been proven to cross the blood-brain barrier (Yang et al. 2005; Zhu et al. 2014) and has the ability to attenuate cerebral edema, decrease the inflammatory response, promote energy homeostasis, and influence synaptic plasticity following TBI (Sharma et al. 2009; Sharma et al. 2010; Wu et al. 2011) and cerebral ischemia/reperfusion injury (Zhao et al. 2008). Therefore, it has been proposed for the treatment of various neuroinflammatory and neurodegenerative conditions of the central nervous system (CNS). To our knowledge, the molecular mechanism underlying curcumin effects remains unclear. In vivo (Zhao et al. 2008; Li et al. 2016) and in vitro (Gonzalez-Reyes et al. 2013; Zhao et al. 2013) evidences demonstrate curcumin is an effective activator of Nrf2; however, it is unknown whether the neuroprotective role of curcumin in TBI is through the Nrf2 pathway.

Considering curcumin has the potential to protect brain damage, and Nrf2 plays a neuroprotective role in TBI-induced secondary injury, we examined whether curcumin enhances the expression of Nrf2 and ARE-regulated antioxidant response in mice subjected to TBI. Further, we explored TBI-induced neuronal apoptosis and inflammatory response in *Nrf2*-KO mice treated with curcumin or not. The results showed that curcumin protected the brain by alleviating brain edema, neuronal apoptosis and inflammation after TBI, partly through an Nrf2-dependent pathway.

2. Materials and methods

2.1. Animals

Male C57BL/6 (wild-type, *WT*) (Charles River Laboratories) and *Nrf2* gene knockout (*Nrf2*-KO) (on C57BL/6 background, originally generated by Dr. Masayuki Yamamoto) mice (8–12 weeks; 20–26 g) were used in this study. The animals were housed at constant temperature and humidity under a 12-hour light/dark cycle, with water and food provided ad libitum. *Nrf2*-KO mice were genotyped by PCR amplification of genomic DNA extracted from tail snips (supplementary data A). The *Nrf2* mRNA level in mice brain was also conducted by qRT-PCR (supplementary data B). The protocols for animal experiments were approved by the China Medical University Animal Care and Use Committee.

2.2. Mouse model of experimental traumatic injury and curcumin administration

TBI was induced according to the Feeney weight-drop contusion model, with slight modifications (Roof et al. 1996). Mice were anesthetized with intraperitoneal sodium pentobarbital (40 mg/kg, i.p.) and placed on a stereotaxic frame. Body temperature was maintained at 37 °C during the surgical procedure. Then, a 4 mm craniotomy was performed over the left parietal cortex to expose the dura, with the center of the opening located 2.5 mm lateral to the sagittal suture and 2.5 mm posterior to the bregma. Considerable care was taken to avoid dural tears and bone flap reinsertion. A weigh-drop device was placed over the dura, with an impact transducer (foot plate) adjusted to stop at a depth of 1 mm below the dura. Then, a 40 g weight was dropped from 20 cm above the dura through a guide tube onto the foot plate. After injury, the skin was closed tightly. Mice in the sham operation group were subjected to the same surgical procedure, including craniotomy, but without cortical impact.

Mice were assigned to three groups, including the sham group with no curcumin treatment (Sham), TBI group with Vehicle treatment (TBI + Veh) and TBI group with curcumin treatment (TBI + Cur). In each group, the mice were sacrificed at 24 h following surgery. The TBI + Cur and TBI + Veh groups were administered curcumin (sigma, St Louis, MO, USA) dissolved in dimethyl sulfoxide (DMSO) (50 mg/kg body weight) or equal volumes of vehicle, intraperitoneally 15 min post TBI. The delivery route, dosing and treatment regimen for curcumin were based on a previous work (Zhao et al. 2008).

A total of 72 *WT* and 54 *Nrf2*-KO mice were assessed, which six mice randomly included in each group. Investigators were blinded to treatment groups during experimental assays and data analysis according to published guidelines.

2.3. Brain water content measurement

Brain water content was quantified using the wet–dry method at 24 h following TBI. Four-mm coronal tissue sections of the ipsilateral cortex centering on the impact site were obtained and immediately weighed to determine the wet weight. The sections were subsequently dried at 65 °C for 72 h to obtain the dry weight. Brain water content (%) was calculated as (wet brain weight − dry brain weight) / wet brain weight × 100%.

2.4. Tissue processing and histology

At 24 h post TBI, mice were deeply anesthetized (100 mg/kg sodium pentobarbital) and perfused via the left ventricle with cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) at 4 °C. Brains were dissected and post-fixed with 4% PFA at 4 °C overnight. The tissues were then dehydrated with ethanol, cleared in xylene, and paraffin embedded. Serial section (4 μm) were obtained, followed by immunofluorescence. Fluoro-Jade C (FJC) staining (n = 6/group) and terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL; n = 6/group) were performed. All sections were visualized under a Leica DMi8 fluorescence microscope (Leica Germany). Mice providing samples for Western blot (n = 6/group) and qRT-PCR (n = 6/group) were perfused with cold PBS only, and the ipsilateral cortex was collected.

2.5. Immunofluorescence

Paraffin-embedded sections were dewaxed and hydrated, followed by incubation in 10% normal horse serum in 1 × PBS. Then, the sections were incubated overnight at 4 °C in one of the following primary antibodies diluted in normal horse serum containing 0.1% tween 20 in PBS: rabbit anti-Tuj1 (1:1000; Abcam, ab78078), rabbit anti-MPO (1:200; Proteintech, 22225-1-AP), rabbit anti-IBA1 (1:1000; Wako, 019-19741), and rabbit anti-Nrf2 (1:200; Santa Cruz Biotechnology, sc-13032). Antigen retrieval was performed by incubating the sections for 5 min in citrate buffer at 95 °C. After several PBS rinses, the sections were incubated with secondary antibodies for 2 h at room temperature. The specificity of immunofluorescence reactions was evaluated by replacement of the respective primary antibody with PBS. Images were used at the same magnification of 400 fold in coronal brain sections. Six sections at the same location from each mouse were selected to compare, and ten random fields without overlaps in each section were evaluated.

2.6. Western blot

Protein (n = 6/group) was extracted from the ipsilateral cortex at the coronal section surrounding the major area of injury with a protein extraction kit. The lysate was cleared by centrifugation at 12000*g* and

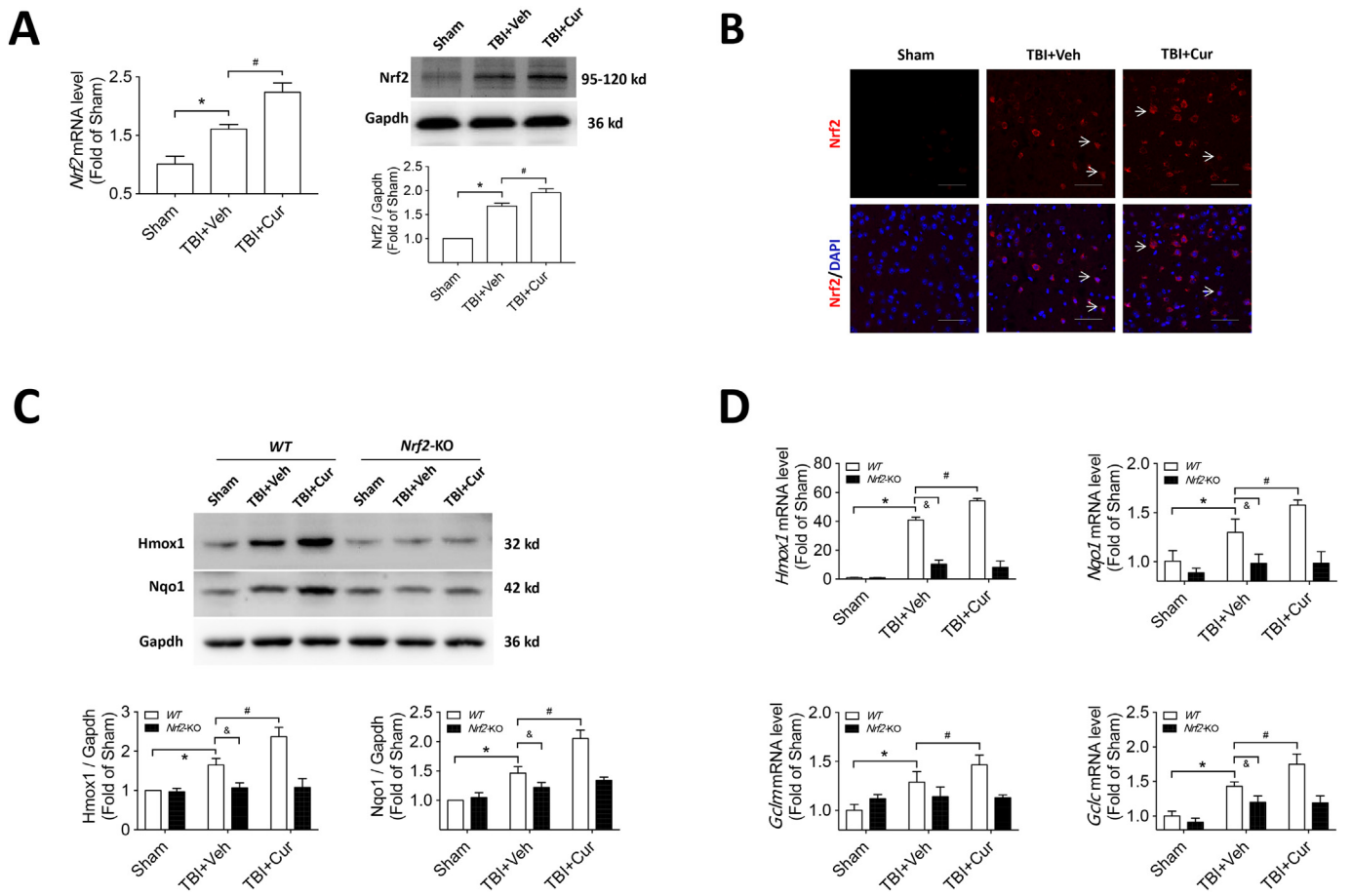

Fig. 1. Curcumin increases Nrf2 expression, promotes nuclear translocation of Nrf2 and upregulates its down-regulated genes in the Nrf2-dependent pathway. (A) The mRNA and protein levels of Nrf2 in the wound site cortex were evaluated by qRT-PCR and Western blot, respectively. Left, mRNA levels; right, representative immunoblots. Relative band densities were quantified in the right lower panel. Data are mean ± SD, $^{*}p < 0.05$ vs. Sham group, $^{\#}p < 0.05$ vs. TBI + Veh (n = 6). (B) Representative immunofluorescent micrographs of ipsilateral cortex after staining with Nrf2 primary antibody in different groups 24 h post injury. Red fluorescence, Nrf2-positive cells; white arrow, nuclear translocation of Nrf2 after TBI with or without curcumin treatment. Scale bar, 50 μm. (C) The representative immunoblots (upper panel) and relative densities (lower panel) of Hmox1 and Nqo1 in the pericontusional cortex at 24 h after TBI. $^{*}p < 0.05$ vs. Sham group, $^{\#}p < 0.05$ vs. TBI + Veh, & $p < 0.05$ vs. wild-type (*WT*) mice (n = 6). (D) The mRNA levels of Nrf2-regulated genes in response to TBI and curcumin in wild-type (*WT*) and *Nrf2*-knockout (*Nrf2*-KO) mice. The target genes were analyzed by qRT-PCR at 24 h. Data are mean ± SD, $^{*}p < 0.05$ vs. Sham group, $^{\#}p < 0.05$ vs. TBI + Veh, & $p < 0.05$ vs. wild-type (*WT*) mice (n = 6). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4 °C for 15 min. The protein concentration was quantified with a BCA assay kit (Beyotime, P0009). Western blotting was carried out as previously described (Zhao et al. 2011). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad). The membranes were blocked with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.1% Tween 20, and probed with primary antibodies against Nrf2 (1:500; Santa Cruz Biotechnology, sc-13032), Hmox1 (1:1000; Proteintech, 10701-1-AP), Nqo1 (1:1000; Proteintech, 11451-1-AP), and cleaved caspase-3 (1:1000; Cell Signaling Technology, 9661), Bcl-2 (1:500; Abcam, ab7973), Gapdh (1:4000; Santa Cruz Biotechnology, SC-25778) at 4 °C overnight. The membranes were washed and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (1:4000; Santa Cruz Biotechnology) for 2 h. Quantification of immunoreactive bands was carried out with the Image J software.

2.7. Fluoro-Jade C (FJC) staining

Fluoro-Jade C (FJC) is a polyanionic fluorescein derivative that stains all degenerating neurons, regardless of the mechanism of cell death. FJC staining of brain sections was performed according to the instructions provided by the manufacturer. The sections were first deparaffinized through two 10 min incubations with xylene and rehydrated by graded alcohol. The sections were then incubated in 0.06% potassium permanganate solution for 10 min, rinsed with distilled water for 2 min and incubated in a 0.0001% solution of FJC (Histo-Chem Inc.) dissolved in 0.1% acetic acid.

2.8. TUNEL staining

Apoptosis was assessed using a TdT-mediated dUTP nick end labeling (TUNEL) detection kit (Beyotime, C1086) according to the manufacturer's instructions. Briefly, the sections were deparaffinized with xylene and dehydrated with graded ethanol. Then, the sections were digested for 15 min in proteinase K (Beyotime, ST533). After several washes with PBS, the sections were incubated with 40 μl of the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and FITC-labeled dUTP for 2 h at 37 °C. Neuronal nuclei were stained with DAPI. Apoptotic index was used to demote the extent of brain damage, defined as the average percentage of TUNEL-positive cells in ten cortical microscopic fields (× 400) in each section. The final average of the percentage of TUNEL-positive cells in the six sections was considered.

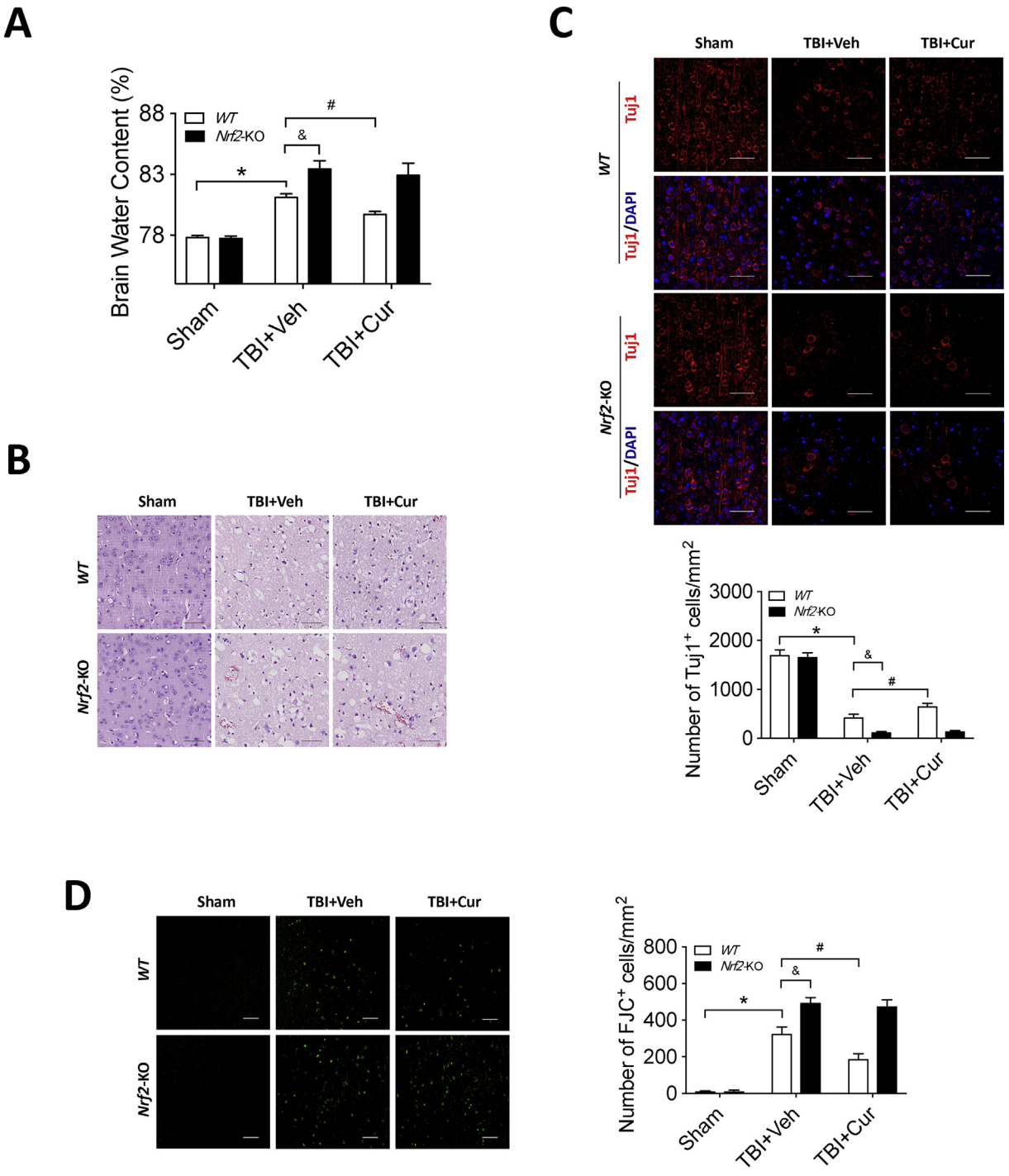

Fig. 2. Curcumin protects mice against secondary brain injury after TBI, but fails to display neuroprotective effects against TBI in *Nrf2*- knockout mice.
(A) Brain water content in the ipsilateral cortex using the wet-dry method at 24 h following TBI. Data are mean ± SD, $^{*}p < 0.05$ vs. Sham group, $^{\#}p < 0.05$ vs. TBI + Veh, $^{\&}p < 0.05$ vs. *WT* mice (n = 6). (B) Representative H&E staining images in the pericontusional area after TBI in *WT* and *Nrf2*-KO mice. Scale bar, 50 μm. (C) Representative neuron specific Tuj1 immunofluorescent staining in pericontusional cortex sections from *WT* and *Nrf2*-KO mice. Tuj1(+) cells were counted and analyzed at a magnification of 400. Data are mean ± SD, $^{*}p < 0.05$ vs. Sham group, $^{\#}p < 0.05$ vs. TBI + Veh, $^{\&}p < 0.05$ vs. *WT* mice (n = 6). Scale bar, 50 μm. (D) Representative Fluoro-Jade C (FJC) staining in pericontusional cortex sections from *WT* and *Nrf2*-KO mice. Degenerated neuronal cells were labeled with green fluorescence, and FJC(+) cells were counted at a magnification of 400 (right panel). Data are mean ± SD, $^{*}p < 0.05$ vs. Sham group, $^{\#}p < 0.05$ vs. TBI + Veh, & $p < 0.05$ vs. *WT* mice (n = 6). Scale bar, 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.9. Gene expression assessment

Total RNA extraction (n = 6/group), reverse transcription, and quantitative PCR were performed as detailed elsewhere (Zhao et al. 2011). Primer sequences are shown in supplementary data C. To ensure that equal amounts of cDNA were added to the PCR reactions, the housekeeping gene *Gapdh* was amplified. Data were analyzed by the ΔΔCT method, with *Gapdh* as a reference gene. All PCR reactions were performed in triplicate.

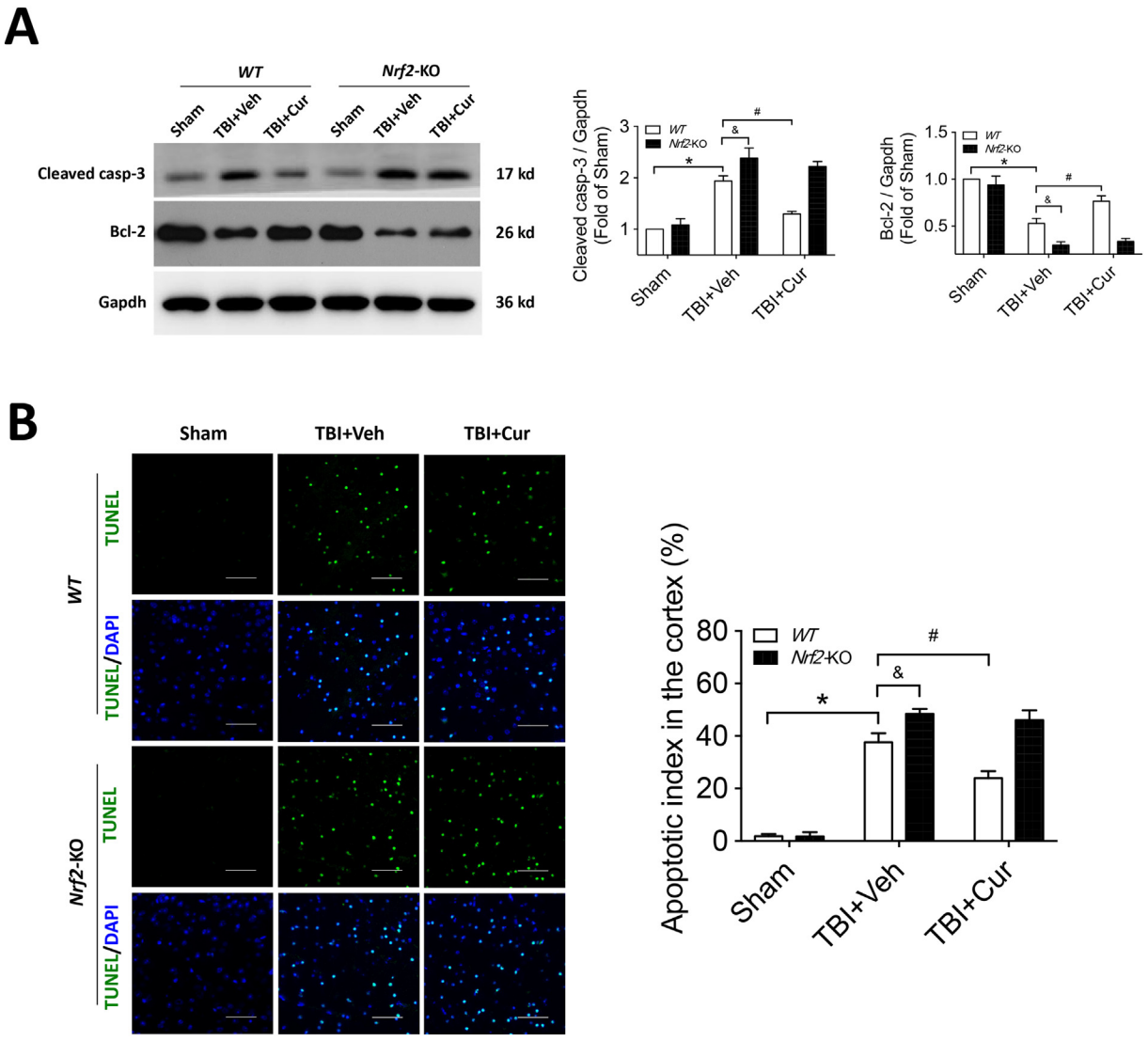

Fig. 3. Curcumin inhibits TBI-induced apoptosis in *WT* but not in *Nrf2*-KO mice.
(A) Representative immunoblots (left panel) and densitometric quantification (right panel) for apoptosis-related proteins. Data are mean ± SD, $^{*}p < 0.05$ vs. Sham group, $^{\#}p < 0.05$ vs. TBI + Veh, $^{\&}p < 0.05$ vs. *WT* mice (n = 6). (B) Representative micrographs for TUNEL and DAPI (4′,6′-diamidino-2-phenylindolle) staining in the pericontusional site at 24 h post-trauma (left panel). The cell apoptotic index was calculated as the number of TUNEL(+) nuclei divided by the total number of DAPI(+) nuclei (right panel). Data are mean ± SD, $^{*}p < 0.05$ vs. Sham group, $^{\#}p < 0.05$ vs. TBI + Veh, $^{\&}p < 0.05$ vs. *WT* mice (n = 6). Scale bar, 50 μm.

2.10. Measurement of malondialdehyde (MDA) level

The MDA content was measured using a commercially available kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturers' instructions. Briefly, at 24 h after TBI, mice were perfused with cold PBS only, and the ipsilateral cortex was collected. Tissue samples were homogenized in nine volumes (grams per liter) of ice-cold saline and centrifuged at 12000*g* for 15 min at 4 °C. The supernatant was used to measure the MDA content using a spectrophotometer. The total protein concentrations were quantified with a BCA assay kit (Beyotime, P0009). The content of MDA was expressed as nmol/mg protein.

2.11. Statistical analysis

Positive cells were counted under a fluorescence microscope by an investigator blinded to grouping. Analyses were performed using SPSS 13.0. All data were presented as mean ± standard deviation (SD). One-way ANOVA was employed to compare differences among the Sham, TBI + Veh and TBI + Cur groups. Bonferroni post hoc analysis was used to determine where the differences occurred. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Curcumin increases the expression and nuclear translocation of Nrf2 and detoxifying enzymes after TBI

Nrf2 mRNA and protein levels in the injured ipsilateral cortex were increased significantly in the TBI groups compared with sham animals; they were further higher in curcumin-treated mice than in vehicle-treated mice at 24 h post-trauma ($p < 0.05$, n = 6/group) (Fig. 1A). Immunofluorescence staining revealed that Nrf2 expression was increased, with translocation into the nucleus around the wound site at 24 h post-trauma (Fig. 1B). Next, Western blot was used to determine the protein levels of antioxidant enzymes after curcumin administration. Hmox1 and Nqo1 levels were increased after TBI ($p < 0.05$, n = 6/group) compared with those of sham animals, and further increased by exposure to curcumin ($p < 0.05$, n = 6/group, Fig. 1C) compared with levels of the TBI + Veh group. Nrf2 deletion attenuated

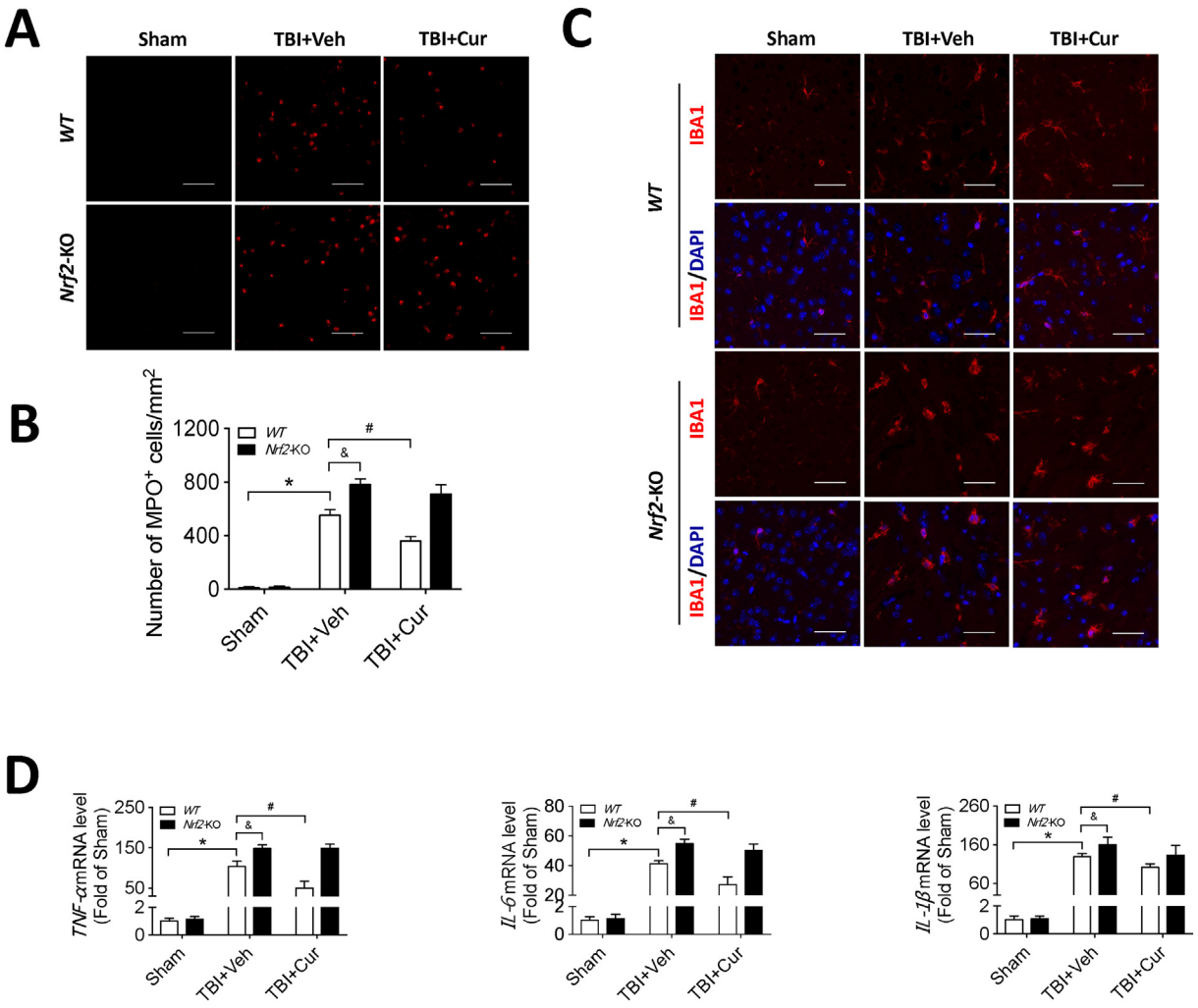

Fig. 4. Curcumin alleviates TBI-induced inflammatory response, but with reduced inhibitory role in *Nrf2*-KO mice.
(A) Representative micrographs of MPO staining of the pericontusional area in the brain cortex. (B) MPO(+) cells were counted in *WT* and *Nrf2*-KO mice at a magnification of 400. Data are mean ± SD, $^{*}p < 0.05$ vs. Sham group, $^{\#}p < 0.05$ vs. TBI + Veh, $^{\&}p < 0.05$ vs. *WT* mice, (n = 6). (C) Representative micrographs for immunostaining of IBA1 in the ipsilateral cortex of *WT* and *Nrf2*-KO mice with or without curcumin treatment. IBA1(+) cells represented activated microglia. Scale bar, 50 μm. (D) Expression levels of proinflammatory mediators in injured ipsilateral cortex specimens from *WT* and *Nrf2*-KO mice assessed by qRT-PCR. Data are mean ± SD, $^{*}p < 0.05$ vs. Sham group, $^{\#}p < 0.05$ vs. TBI + Veh, $^{\&}p < 0.05$ vs. *WT* mice, (n = 6).

the upregulation of Hmox1 and Nqo1 levels in TBI + Veh and even the TBI + Cur groups ($p < 0.05$, n = 6/group) (Fig. 1C). In addition, transcription levels of genes downstream of Nrf2 were detected by real-time PCR; *Hmox1, Nqo1, Gclc,* and *Gclm* levels were increased in the TBI + Veh group compared with sham animals. Specifically, Hmox1 was increased by approximately 40-fold after TBI (Fig. 1D). The downstream genes of Nrf2 were further increased when exposed to curcumin in the TBI + Cur group ($p < 0.05$, n = 6/group). Meanwhile, Nrf2 deletion attenuated TBI-induced upregulation of antioxidant enzymes downstream of Nrf2 in TBI + Veh treated animals and even the TBI + Cur group ($p < 0.05$, n = 6/group) (Fig. 1D).

3.2. *Nrf2-knockout mice exhibit less response to curcumin induced neuroprotective roles in TBI*

To assess the protective role of curcumin on BBB (Blood Brain Barrier) leakage, the brain water content of the ipsilateral cortex was assessed. As shown in Fig. 2A, brain water content was significantly decreased in curcumin-treated mice compared with vehicle-treated animals at 24 h post-trauma ($p < 0.05$) (Fig. 2A). However, curcumin did not reduce water content in the ipsilateral brain from *Nrf2*-KO mice ($p > 0.05$) (Fig. 2A). To explore the neuronal protective role of curcumin and Nrf2 function, H&E staining was performed. As shown in Fig. 2B, curcumin improved the survival of neuronal cells and alleviated TBI-caused edema, as reflected by the intercellular space in the impacted site, which was not obvious in mice with Nrf2 deletion. To further explore the role of curcumin in neuron survival after TBI, immunostaining with Tuj1, a neuron specific biomarker, was carried out. As shown in Fig. 2C, the number of Tuj1(+) cells was increased after curcumin administration at 24 h post-injury after TBI compared with that in the TBI + Veh groups ($p < 0.05$, n = 6, Fig. 2C). Nrf2 deletion reduced the protective effects of curcumin on neuron survival (Fig. 2C). We also performed FJC staining to explain the degenerated cells in the ipsilateral cortex after TBI. The results showed that curcumin prevented TBI-induced neuronal degeneration, at least in part, through the Nrf2 pathway, with the evidence that markedly more positive cells were found post-injury, while curcumin administration did not enhance TBI-induced neuronal degeneration in *Nrf2*-KO mice (n = 6, Fig. 2D).

3.3. *Curcumin exhibits anti-apoptotic effects on TBI-induced neuronal damage through the Nrf2 pathway*

To explore whether curcumin affects neuronal apoptosis after cortex injury, the protein levels of cleaved caspase-3 and the anti-apoptotic

protein Bcl-2 were assessed in the ipsilateral cortex. As shown in Fig. 3A, curcumin downregulated TBI-induced increase of cleaved caspase-3 and upregulated Bcl-2 level, compared with the TBI + Veh group in *WT* mice ($p < 0.05$). Nrf2 deletion aggravated the level of cleaved caspase-3 and attenuated expression of Bcl-2 in the ipsilateral cortex post-trauma compared to *WT* animals ($p < 0.05$). Next, densitometric quantification of the immunoblots was performed (Fig. 3A, right panel). To evaluate apoptosis in the pericontusional area of the ipsilateral cortex, in situ terminal transferase d-UTP nick-end labeling (TUNEL) assay was conducted. As shown in Fig. 3C, TUNEL(+) cells were evident 24 h post-trauma in the pericontusional tissue. The apoptotic index (AI) decreased significantly in the curcumin treatment group compared with vehicle treated animals ($p < 0.05$). Quantitative analysis indicated that mice with Nrf2 deletion showed higher apoptotic index post-trauma than *WT* animals ($p < 0.05$). There was almost no protective effect of curcumin on apoptotic index in *Nrf2*-KO mice compared with *WT* mice ($p > 0.05$) (Fig. 3B).

3.4. Curcumin does not inhibit the inflammatory response in Nrf2-knockout mice

To explore the effect of curcumin on TBI-induced inflammatory response, we first examined neutrophil infiltration by MPO immunostaining around the lesion at 24 h post-TBI. Curcumin decreased the number of MPO(+) cells compared with the vehicle in the TBI model ($p < 0.05$, n = 6/group; Fig. 4A, upper panel). Mice with Nrf2 deletion exhibited more MPO positive cells in the injured ipsilateral cortex than compared with *WT* animals, with reduced inhibitory effects of curcumin on inflammation in *Nrf2*-knockout animals ($p < 0.05$, n = 6/group; Fig. 4A, lower panel). As shown in Fig. 4B, the numbers of MPO positive cells around the lesion site showed a significant difference between the TBI + Veh and TBI + Cur groups ($p < 0.05$, n = 6/group). Next, due to the contribution of microglial in traumatic brain injury (TBI), we detected activated microglia by microglia specific antibody IBA1 immunostaining. In the pericontusional tissue of sham mice, a few quiescent microglia were found with small cell bodies and fine appearance. Microglia/macrophages were rapidly increased by IBA1 immunostaining and exhibited a deramified and hypertrophic morphology after TBI. Administration of curcumin ameliorated these morphological changes (Fig. 4B, upper panel). Although no overt difference in the number of IBA1(+) cells between *WT* and *Nrf2*-KO mice was found (Fig. 4B, lower panel), the microglia were indeed clustered together and exhibited a deramified and hypertrophic morphology with or without curcumin in *Nrf2*-KO mice (Fig. 4B, lower panel). Finally, the mRNA levels of proinflammatory mediators in the ipsilateral cortex of *WT* and *Nrf2*-KO mice were detected by qRT-PCR. Nrf2 knockdown increased the levels of proinflammatory cytokines induced by TBI, e.g. *TNF-α*, *Interleukin-6 (IL-6)* and *Interleukin-1β (IL-1β)* (Fig. 4D). Exposure to curcumin downregulated these proinflammatory cytokines in *WT* mice after TBI, while no significant differences in *TNF-α*, *IL-6* and *IL-1β* levels were found with or without curcumin treatment in *Nrf2*-KO mice (Fig. 4D).

3.5. Curcumin reduces TBI-induced oxidative damage in ipsilateral cortex through Nrf2 pathway

To investigate the effect of curcumin on oxidative damage, MDA was measured in the ipsilateral cortex at 24 h post TBI. As shown in supplementary data D, MDA level was elevated in the injured cortex ($p < 0.05$) and curcumin attenuated the change in *WT* mice ($p < 0.05$). MDA level in *Nrf2*-KO mice was higher than that in *WT* mice ($p < 0.05$), which was not decreased significantly with curcumin administration (Supplementary data D).

4. Discussion

Secondary brain injury occurs within hours to days after TBI. There are several important pathological processes responsible for secondary damage in TBI, including oxidative stress, inflammatory reactions, apoptosis and glutamate excitotoxicity. Considering that curcumin, a popular antioxidant, has a neuroprotective role in TBI (Wu et al. 2006; Zhu et al. 2014), we explored the underlying mechanism by which curcumin protects the brain from TBI-induced oxidative insults. Emerging evidence demonstrates that curcumin enhances Nrf2 activation in the brain (Cui et al. 2017; Tu et al. 2017). In the present study, the neuroprotective roles of curcumin following TBI in mice were assessed. The results provided substantial evidence that intraperitoneal post-injury treatment with curcumin improved the expression and nuclear translocation of Nrf2, and upregulated its downstream antioxidant enzymes such as Nqo1, Hmox1, Gclc and Gclm. Treatment with curcumin alleviated cerebral edema and oxidative damage, suppressed neuronal apoptosis and relieved acute inflammatory reactions induced by TBI. *Nrf2* suppression attenuated the neuroprotective effects of curcumin. The present results provided substantial evidence that Nrf2 signaling may be an important mechanism through which curcumin improves the outcome following TBI.

Several studies have demonstrated that oxidative stress is one of the major causes of secondary injury (Cornelius et al. 2013; Tumer et al. 2013), and Nrf2 activation might attenuate the secondary damage following TBI (Lu et al. 2015). Multiple reports provide evidence that Nrf2 alters not only oxidative stress, but also apoptosis and inflammation in TBI via different molecules and pathways. In the present study, curcumin displayed neuroprotective effects by improving the nuclear translocation of Nrf2 and upregulating antioxidant enzymes in the Nrf2-ARE pathway, e.g. heme oxygenase-1 (HO-1), NADPH quinine oxidoreductase-1 (NQO-1), GCLC and GCLM, suggesting that curcumin effects were partly through the Nrf2 regulated antioxidant response. It was reported that apoptotic cells are detectable in the contusion zone after a minimum posttraumatic interval of 110 min (Dressler and Vemuganti 2009). In this study, curcumin decreased the number of TBI-induced apoptotic cells and ameliorated neuron survival in the ipsilateral contusion zone, consistent with previous findings (Wu and Liu 2016). Treatment of curcumin upregulated mitochondrial death pathway-related members B-cell lymphoma-2 (Bcl-2) and downregulated the apoptosis executioner cleaved caspases-3 in ipsilateral cortex, suggesting the anti-apoptotic effect of curcumin is closely associated with the mitochondrial apoptotic pathway, which might also be regulated by Nrf2.

Neuroinflammation may be a significant contributor to TBI-induced substantial neural damage and behavior impairment. Though no difference in the number of activated microglia was found after Nrf2 deletion, the inflammatory response was much more pronounced after TBI in the present study. Numerous evidences show that Nrf2 regulates neuroinflammation, since LPS induces a much more pronounced neuroinflammatory response in Nrf2-deficient mice compared with normal animals (Innamorato et al. 2008). In addition, increased expression levels of proinflammatory markers, such as cyclooxygenase-2 (COX-2), inducible nitric oxide synthases (iNOS), IL-6, tumor necrosis factor-a (TNF-a), and ICAM-1, are found in Nrf2 deficient animals after TBI (Jin et al. 2008). Overexpression of Nrf2 resulted in reduced phagocytic ability and expression levels of TNF-αand IL-6 in microglia, suggesting Nrf2 protects against TBI, at least in part, by regulating microglial function (Wu and Liu 2016). The counteracting effect of curcumin on TBI-induced inflammatory response was regulated partly by Nrf2 in the current study, while a recent report showed that curcumin inhibits the TLR4/MyD88/NF-κB signaling pathway (Zhu et al. 2014). Although the relationship between NF-κB and Nrf2 is not defined, the identification of NF-κB binding sites in the promoter region of the Nrf2 gene suggests a crosstalk between these two regulators in the inflammatory process (Nair et al. 2008). Multiple studies showed that Nrf2 regulates

neuroinflammatory responses, while enhanced inflammation also blocks the Nrf2 system by inducing the activation of specific kinases and epigenetic modifications (Guo et al. 2015). The relationship between Nrf2 and inflammation deserves further investigation.

Many neuroprotective drugs attenuate TBI-induced secondary injury via Nrf2 activation (Xu et al. 2014; Miller et al. 2015; Shu et al. 2016; Zhang et al. 2017). A number of Nrf2 inducers have been tested clinically. Indeed, sulforaphane (www.ClinicalTrials.gov; NCT01716858; NCT01474993), dimethyl fumarate (DMF) (www.ClinicalTrials.gov; NCT01930708) and triterpenoids have been reported to protect the brain from oxidative stress via NRF2-dependent mechanisms in clinical studies (Gold et al. 2012; Scannevin et al. 2012; Kaidery et al. 2013). Due to its numerous pharmacological activities, low toxicity and widespread availability (Ravindranath and Chandrasekhara 1980; Shoba et al. 1998; Pan et al. 1999), curcumin has been applied to increase cell survival, decrease the brain water content, attenuate microglia/macrophage infiltration, and decrease inflammatory mediator release in the injured tissue after TBI (Laird et al. 2010; Zhu et al. 2014). The present study showed that post-injury treatment with curcumin indeed played a neuroprotective role in TBI, at least partly through the Nrf2 signaling pathway, suggesting the therapeutic potential of curcumin based on Nrf2 signaling. The current findings support the necessity for assessing the application of curcumin for TBI treatment, also suggesting that modulation of Nrf2 signaling in neurons and microglia/macrophages might constitute a potential therapeutic target in TBI.

This present study used the global *Nrf2*-KO mice to illustrate the protective role of curcumin against TBI-induced secondary injury. It could not be avoided that the ablation of Nrf2 in other organs or tissues might take some side-effects on the pathogenisis of secondary injury after TBI. It is necessary to further confirmed our results in the nervous conditional knockout mice. In addition, we only explored the neuroprotective roles of curcumin through Nrf2-regulated oxidative stress, apoptosis and inflammation after TBI. Recent studies also provide evidence that Nrf2 transcriptionally modulates autophagy (Pajares et al. 2016; Zhang et al. 2017), the ubiquitin-proteasome system (UPS) (Ding et al. 2017), and other neuroprotective proteins, such as brain derived neurotrophic factor (Sakata et al. 2012), the mitochondrial transcription (co)-factors NRF-1 and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1a) (Piantadosi et al. 2011) through ARE binding sites. Further work will be needed to determine if the neuroprotective role of curcumin might be related to other Nrf2-regulated pathways.

5. Conclusion

The effect of curcumin on TBI brain is associated with the activation of Nrf2 signaling, which highlights the neuroprotective role of Nrf2 and the potential therapeutic use of curcumin in TBI.

Conflicts of interest

The authors declare that there is no completing interests.

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