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Edaravone attenuates traumatic brain injury through anti-inflammatory and anti-oxidative modulation

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Abstract. Traumatic brain injury (TBI) is among the leading causes of irreversible neurological damage and death worldwide. The aim of the present study was to investigate whether edaravone (EDA) had a neuroprotective effect on TBI as well as to identify the potential mechanism. Results demonstrated that EDA suppressed inflammatory and oxidative responses in mice following TBI. This was evidenced by a reduction in glutathione peroxidase, interleukin 6, tumor necrosis factor-α and hydrogen peroxide levels, in addition to an increase in hemeoxygenase-1, quinone oxidoreductase 1 and superoxide dismutase levels, thereby mitigating neurofunctional deficits, cell apoptosis and structural damage. EDA prevented the transfer of NF-κB protein from the cytoplasm to the nucleus, whilst promoting the expression of nuclear factor erythroid 2-related factor 2 (Nrf2) protein in mice following TBI. These results indicated that EDA exerted neuroprotective effects, including impeding neurofunctional deficits, cell apoptosis and structural damage, in mice with TBI, potentially via suppression of NF-κB-mediated inflammatory activation and promotion of the Nrf2 antioxidant pathway.

Introduction

Traumatic brain injury (TBI) is a serious neurological condition (1,2). Millions of people suffer from TBI-related disabilities worldwide, costing billions of dollars annually (3). TBI-related nerve injury is attributed to both primary and secondary injury mechanisms (4,5). Primary injury manifests as immediate damage, whilst secondary injury is a long-term process, accompanied by oxidative stress, neuronal apoptosis and an inflammatory response (2,6-8), ultimately leading to neuronal death. There is growing evidence that inflammation and oxidative stress serve pivotal roles in the course of secondary brain injury (10-12). Thus, anti-inflammatory treatments that combat oxidative stress are predicted to be effective therapeutic strategies for improving outcomes following TBI.

The inflammatory response and oxidative stress are important adverse pathological events that occur following TBI. It is widely accepted that NF-κB performs an essential role in the inflammatory response, triggering the expression of tumor necrosis factor (TNF), pro-inflammatory interleukins (ILs) and interferons in response to inflammation induction (13,14). Nuclear factor erythroid 2-related factor 2 (Nrf2) is an inhibitor of oxidative stress. In unstressed conditions, Nrf2 is retained in the cytoplasm by binding to kelch-like ECH-associated protein 1 (15,16). However, following Nrf2 activation, an increase in the expression of downstream cytoprotective proteins, such as heme oxygenase 1 (HO-1) and quinine oxidoreductase 1 (NQO-1), occurs, enhancing the cell's resistance to oxidative stress (17). In addition, genetic knockout of Nrf2 exacerbates oxidative stress injury following TBI in mice (18). Thus, targeting NF-κB and Nrf2 is an attractive strategy for identifying novel treatments for TBI.

Edaravone (EDA; 3-methyl-1-phenyl-2-pyrazolin-5-one) is a free radical scavenger that has been demonstrated to improve neurological function in mice following cerebral hemorrhage (19). Multiple studies also indicate that EDA exerts a neuroprotective effect mediated by quenching oxidative stress in a model of ischemia/reperfusion injury (20-22). Furthermore, EDA has a protective effect in multiple *in vitro* models of brain function, mediated by the inhibition of oxidative stress. However, few studies have addressed the role of oxidative stress inhibition by EDA in regards to TBI. One study demonstrated that EDA attenuates the inflammatory response in a rat transient focal ischemia model by regulating NF-κB (23). However, the mechanism by which EDA attenuates the inflammatory response in a TBI mouse model remains poorly understood. In addition, it is also not known whether EDA has a role in protecting neurological function following TBI by regulating Nrf2.

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The objective of the present study was to investigate the ability of EDA to attenuate a TBI-induced inflammatory response and oxidative stress injury in mice. The results indicated that EDA likely suppressed the inflammatory response and oxidative stress following TBI by regulating NF-κB and Nrf2. Collectively, these findings suggested that EDA may be an effective novel treatment for TBI.

Materials and methods

Experimental animals. A total of 60 male C57BL/6 mice (20-25 g) were obtained from the Experimental Animal Center of Nanjing Medical University. The animal study protocols were approved by the Animal Care and Use Committee of Wenzhou Medical University. Mice were housed under standard conditions, including adequate temperature, standard humidity and a 12-h light/dark cycle. All the animals were allowed free access to food and water and acclimatized for at least 7 days before any experiment.

Reagents and chemicals. EDA was purchased from the Simcere Pharmaceutical Group. Anti-β-actin (cat. no. 3700s) and anti-NF-κB (cat. no. 8242s) antibodies were purchased from Cell Signaling Technology, Inc. Anti-Nrf2 (cat. no. ab31163) and anti-histone3 (cat. no. ab1791), antibodies were purchased from Abcam. Anti-mouse secondary antibodies and anti-rabbit secondary antibodies were purchased from Multi Sciences Biotech Co.

TBI model. The TBI model was established as previously described (24). In brief, the mice were anesthetized by intraperitoneal injection of chloral hydrate (400 mg/kg) and placed onto a stereotaxic frame (David Kopf Instruments; Fig. 1C). A portable drill was used to penetrate the right parieto-temporal cortex in order to allow removal of the bone flap. A pneumatic cylinder (velocity of 4 m/s; depth, 1 mm) was then used to apply a controlled cortical impact. Following the blow, the scalp was sutured closed, and the mice were returned to cages for recovery. Mice subjected to the same procedures without impact were the sham group. EDA (3 mg/kg) was administered by intraperitoneal injection 1 h post-TBI. This dose of EDA administration was based on previous studies of neuroprotection by EDA in an intracerebral hemorrhage mouse model (9,17). Furthermore, according to our previous studies, inflammation and oxidative stress levels were significantly upregulated following TBI and peaked 24 h post-surgery. Therefore, the 24 h timepoint was selected to examine the anti-inflammatory and anti-oxidative stress capacity of EDA.

Neurological evaluation. The 10-point Neurological severity score (NSS) (24) was used to assess mice 24 h following TBI. NSS tests were as follows: i) Ability and initiative to exit circle of 30 cm diameter with a time limit of 3 min; ii) paresis of upper and/or lower limb of the contralateral side; iii) alertness, initiative and motor ability to walk straight once placed on the floor; iv) innate startle reflex where the mouse bounces in response to a loud hand clap; v) seeking behavior involving physiological behavior as a sign of 'interest' in the environment; vi) ability to balance on a beam of 7 mm width for at least 10 sec; vii) ability to grip on a beam of 5 mm width for at least 10 sec; viii) ability to cross a 30 cm long beam of 3 cm width; ix) ability to cross a 30 cm long beam of 2 cm width; and x) ability to cross a 30 cm long beam of 1 cm width. One point is given for failing to perform each of the tasks.

Hematoxylin and eosin (H&E) and Nissl staining. At 24 h and 7 days following TBI, mice were anesthetized and perfused with saline. Brain tissue was collected and fixed in 4% (w/v) paraformaldehyde (PFA) for 24 h at 4˚C, then embedded in paraffin wax. Sections (5 μm) were mounted on poly-L-lysine-coated slides for histopathological examination. H&E and Nissl staining was then performed according to the manufacturer's instructions. Images were captured by light microscope using a Nikon Eclipse 80i (Nikon Corporation) with Nissl-positive cells automatically counted in five randomly selected fields per sample and quantified using Integrated Performance Primitives v9.0 software (Intel Corporation).

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining. An *in situ* cell death detection kit (cat. no. 1168481710; Roche Diagnostics) was used to determine the presence of apoptotic cells in sections. In brief, the sections (5 μm) were cut from the same blocks as the H&E staining, then were deparaffinized, rehydrated then washed briefly with distilled water before digestion for 15 min using proteinase K at room temperature. The sections were then washed and incubated with a prepared TUNEL reaction mixture in a dark humidifying box for 1 h at 25˚C. The sections were then washed and DAPI was added to stain the cell nuclei, prior to being sealed with a coverslip. Brain slices treated with 10 U/ml DNase I buffer for 10 min at room temperature prior to incubation with the TUNEL reaction mixture provided a positive control for the study. The negative control was brain slices incubated with the TUNEL reagent without the addition of TdT enzyme. Positive cells were identified and analyzed using fluorescence microscopy by an investigator blinded to the experimental groups. TUNEL-positive cells were counted in five randomly selected fields in each sample and quantified using Integrated Performance Primitives v9.0 software (Intel Corporation).

Measurement of superoxide dismutase (SOD), glutathione peroxidase (GPx), hydrogen peroxide (H_2O_2), IL-6 and TNF-α concentration. Cortical tissue was homogenized and centrifuged at 12,000 x g for 15 min. The supernatant was collected for the spectrophotometric measurement of SOD (cat. no. S0109), GPx (cat. no. S0056), H_2O_2 (cat. no. S0038), IL-6 (cat. no. F1083) and TNF-α (cat. no. F1163) using the respective kits (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. In brief, supernatants were added to 96-well plates that had been coated with specific murine monoclonal antibodies raised against SOD, GPx, H_2O_2, IL-6 and TNF-α then incubated for 90 min at 37˚C. Biotin-labeled specific antibodies for SOD, GPx and H_2O_2 were added and incubated for a further 60 min at 37˚C. Finally, 3,3'5,5-tetramethylybenzidine substrate was added and incubated for 25 min at 37˚C. Stock concentrations of murine SOD, GPx and H_2O_2 were serially diluted to create standard titration curves. A microplate reader was used to measure optical densities at 450 nm.

Western blot analysis. Protein extracts were obtained using the Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology), total protein concentration was quantified using the bicinchoninic acid assay kit (cat. no. p0012s; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. A total of 80 μg of protein was loaded per lane, then separated via SDS-PAGE on a 10% of gel. The separated proteins were transferred to polyvinylidene-difluoride membranes (EMD Millipore). After blocking for 2 h at 25˚C in 5% skimmed milk, the membranes were incubated with primary antibodies anti-Nrf2 (1:1,000), anti-histone 3 (1:1,000), anti-β-actin (1:3,000) and anti-NFκB (1:1,000) overnight at 4˚C. The membranes were then washed with Tris-buffered saline and Polysorbate 20 and incubated with secondary antibodies (1:10,000) for 1 h at room temperature. The protein bands were visualized using enhanced chemiluminescence (cat. no. P0018AS; Beyotime Institute of Biotechnology) and the ChemiDicTM XRS+ Imaging System (Bio-Rad Laboratories, Inc.), and the densities of the immunoreactive bands were analysed using Image J software (National Institutes of Health). β-actin was used as the loading control for quantification.

Reverse transcription quantitative PCR (RT-qPCR). Total RNA was extracted from cells and ipsilateral cortex samples with TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). The concentration of total RNA was detected using a nucleic acid protein analyzer (Beckman Coulter, Inc.). To avoid RNA degradation, reverse transcription was performed immediately with the Prime Script RT-PCR kit (Takara Bio, Inc.) according to the manufacturer's instructions. qPCR was performed on the Eppendorf Real Plex 4 instrument (Eppendorf) using real-time SYBR Green (cat. no. 1708882AP; Bio-Rad Laboratories, Inc.). The specific primers designed by Invitrogen (Thermo Fisher Scientific, Inc.) with the following sequences: NQO1 forward, 5'-CATTCTGAAAGGCTGGTTTGA-3' and reverse, 5'-CTAGCTTTGATCTGGTTGTCAG-3'; HO-1 forward, 5'-ATCGTGCTCGCATGAACACT-3' and reverse, 5'-CCAACACTGCATTTACATGGC-3'; TNF-α forward, 5'-TGATCCGCGACGTGGAA-3' and reverse, 5'-ACCGCCTGGAGTTCTGGAA-3'; IL-6 forward, 5'-CCAAGAGGTGAGTGCTTCCC-3' and reverse, 5'-CTGTTGTTCAGACTCTCTCCCT-3' and β-actin forward, 5'-CCGTGAAAAGATGACCCAGA-3' and reverse, 5'-TACGACCAGAGGCATACAG-3'. The thermocycling conditions were as follows: 95˚C for 10 min followed by 40 cycles of 95˚C for 15 sec then 60˚C for 1 min. mRNA levels were quantified using the $2^{-\Delta\Delta Cq}$ method (25).

Immunohistochemistry. Following deparaffinization and rehydration, brain tissue sections and cells were mounted on coverslips and fixed with 4% PFA for 48 h at 4˚C. These samples then were then blocked with 5% bovine serum albumin in PBS for 30 min at 37˚C then incubated overnight at 4˚C with primary antibody anti-NF-κB (1:300). Following washing with PBS (7 min washes for a total of three times), the slides were incubated for 2 h at room temperature with the secondary antibody (1:1,000), using the same antibodies as for western blot analysis. Following washing in PBS, the slides were stained with DAPI for 7 min. Fluorescence was detected using a confocal laser microscope (Nikon Corporation). Analysis was performed by blinded observers using Integrated Performance Primitives v9.0 software (Intel Corporation).

Statistical analysis. Data are presented as the mean ± standard error of the mean with experiments repeated at least three times. GraphPad Pro 5.0 (GraphPad Software, Inc.) was used to process data. Statistical significance was assessed between two groups using student's t-test, and between multiple groups using one-way analysis of variance followed by Dunnett's post hoc test. $P<0.05$ was considered to indicate statistical significance.

Results

EDA treatment decreases neurofunctional deficits in mice following TBI. H&E staining was used to investigate neuronal survival. Compared with the TBI group, smaller lesion cavity volumes were observed following EDA treatment (Fig. 1A). Furthermore, treatment with EDA resulted in marked preservation of the number of Nissl-positive stained neurons in the treatment group compared with the non-treated group (Fig. 1B and D). TUNEL-positive apoptotic cells were present at a higher frequency in the TBI group compared with the sham group, and the apoptotic index of the EDA group was decreased compared with the TBI group (Fig. 1E and F). The neuroprotective role of EDA was investigated 24 h following TBI. The NSS score of mice in the TBI group was significantly higher compared with the EDA group (Fig. 1G).

EDA suppresses the TBI-induced inflammatory response. IL-6 and TNF-α levels in the cortex of brain tissue samples were investigated to determine whether the EDA protective effect was due to its ability to suppress inflammation induced by TBI. IL-6 and TNF-α concentration in brain tissue (Fig. 2A and B) and mRNA expression levels (Fig. 2C and D) were higher in the TBI group compared with the sham group. However, both cytokine concentration and mRNA expression significantly decreased following EDA treatment (Fig. 2).

EDA suppresses oxidative stress levels following TBI. SOD, GPx and H_2O_2 levels in brain cortex samples were investigated to determine whether the EDA protective effect was due to its ability to suppress oxidative stress induced by TBI. H_2O_2 levels were significantly higher in the TBI group compared with the sham group (Fig. 3C), whilst EDA treatment resulted in a significant decrease in levels in the brain (Fig. 3C). The levels of important antioxidant enzymes SOD and GPx were significantly decreased in the TBI group compared with the sham group in the brain. However, SOD and GPx levels were higher in the EDA treated group compared with the TBI group (Fig. 3A and B).

EDA inhibits NF-κB protein translocation from the cytoplasm to the nucleus and upregulates Nrf2 protein expression following TBI. NF-κB activity is essential to induce an inflammatory response and it was demonstrated that EDA treatment significantly decreased inflammatory cytokine expression (Fig. 2). Thus, the effects of EDA on NF-κB activation following TBI were further examined. Following

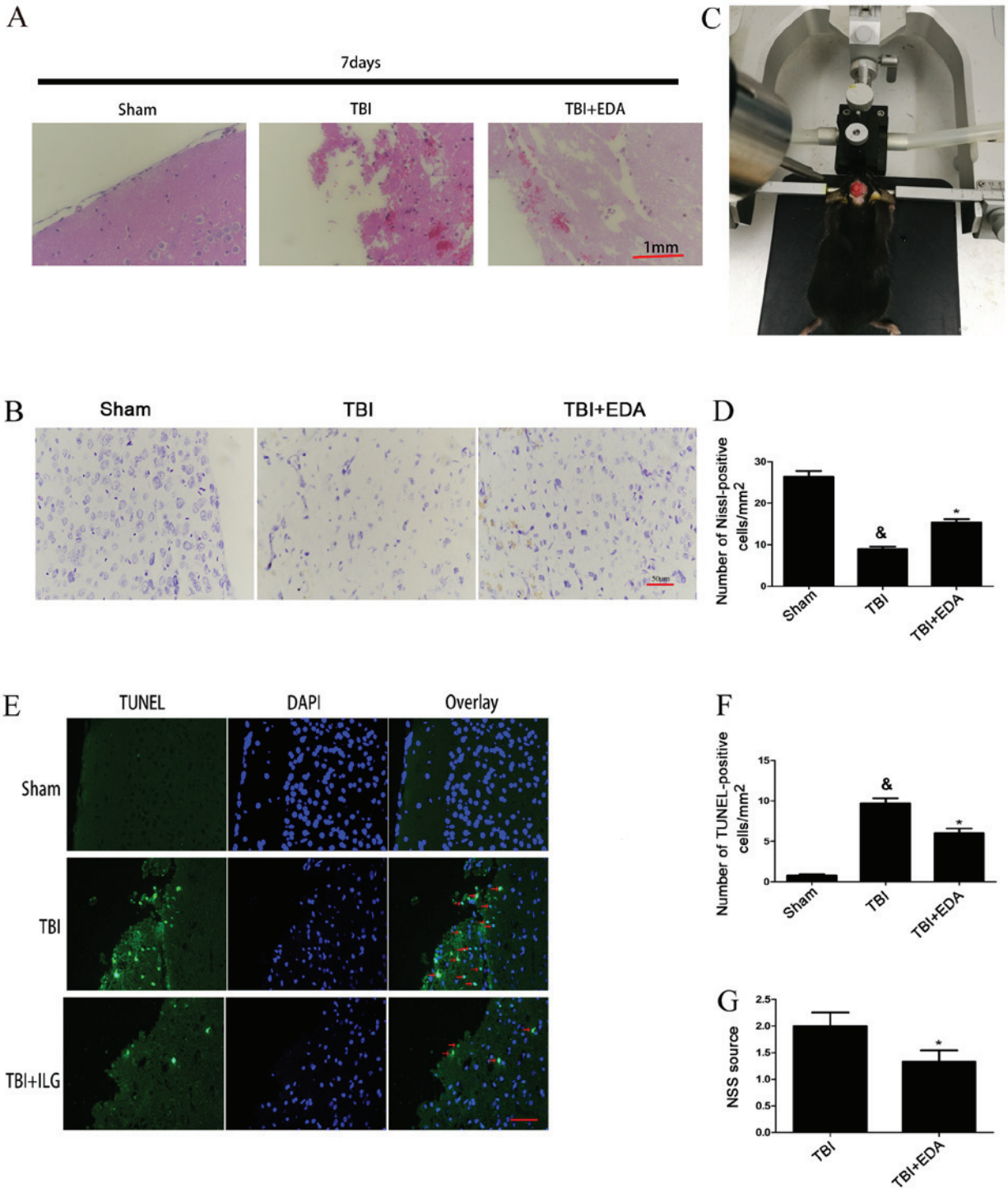

Figure 1. EDA treatment increases the survival of neurons and improves TBI recovery. (A) Hematoxylin and eosin staining of the various groups 7 days following TBI (scale bar, 1 mm; n=5). (B) Nissl staining of the various groups 24 h following TBI (scale bar, 50 μm; n=5). (C) Photo of experimental setup demonstrating a mouse fixed in a stereotaxic frame ready for TBI induction. (D) Quantification of the number of positive Nissl-stained cells in the various groups following TBI (n=5). (E) Immunofluorescence staining of TUNEL (green) in the cortex 24 h post-injury (scale bar, 50 μm). (F) Quantification of TUNEL-positive cells in the various groups following TBI (n=5). (G) NSS analyses of sham, TBI and TBI+EDA groups (n=6). $^{\&}$P<0.05 vs. sham group; *P<0.05 vs. TBI group. EDA, edaravone; TBI, traumatic brain injury; TUNEL, terminal deoxynucleotide transferase dUTP nick end labelling; NSS, neurological severity score.

EDA treatment, reduced expression of NF-κB was detected in the nucleus compared with untreated controls (Fig. 4A and B). This was confirmed by immunofluorescence analysis of NF-κB (Fig. 4C and D). To validate that EDA significantly increased antioxidant enzyme activity following TBI, Nrf2 expression was determined by western blot analysis. Both TBI and EDA treatment induced higher Nrf2 expression compared with the sham group (Fig. 4E). In addition, the EDA-treated group exhibited significantly increased Nrf2 expression compared with the TBI group (Fig. 4F). Due to Nrf2 being activated by EDA, the expression of genes downstream to Nrf2 were investigated. RT-qPCR analysis demonstrated that HO-1 and NQO-1 mRNA expression was significantly increased following TBI compared with the sham group

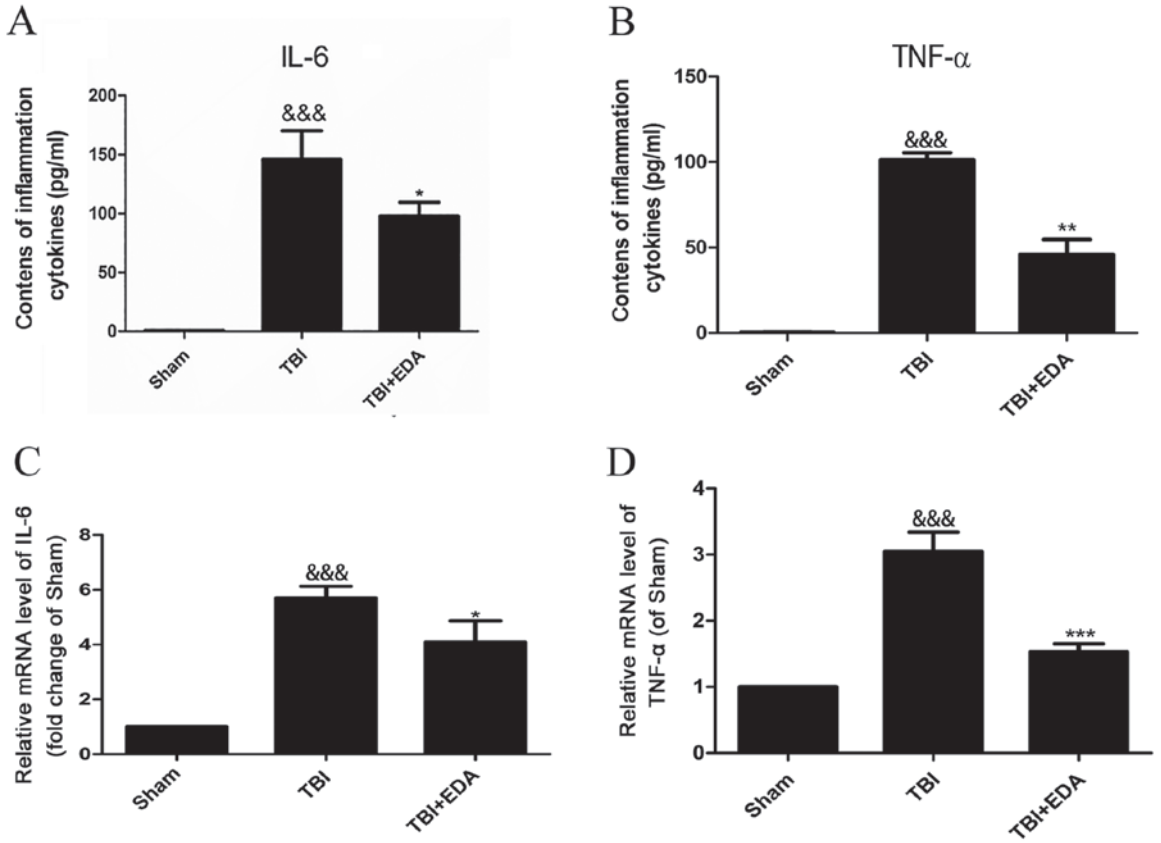

Figure 2. EDA treatment attenuates inflammatory response induced by TBI. (A) IL-6 and (B) TNF-α levels in brain tissue. (C) IL-6 and (D) TNF-α mRNA expression levels in brain tissue determined by reverse transcription-quantitative PCR (n=6). $^{\&\&\&}$P<0.001 vs. sham group; *P<0.05, **P<0.01 and ***P<0.001 vs. TBI group. EDA, edaravone; TBI, traumatic brain injury; IL, interleukin; TNF-α, tumor necrosis factor-α.

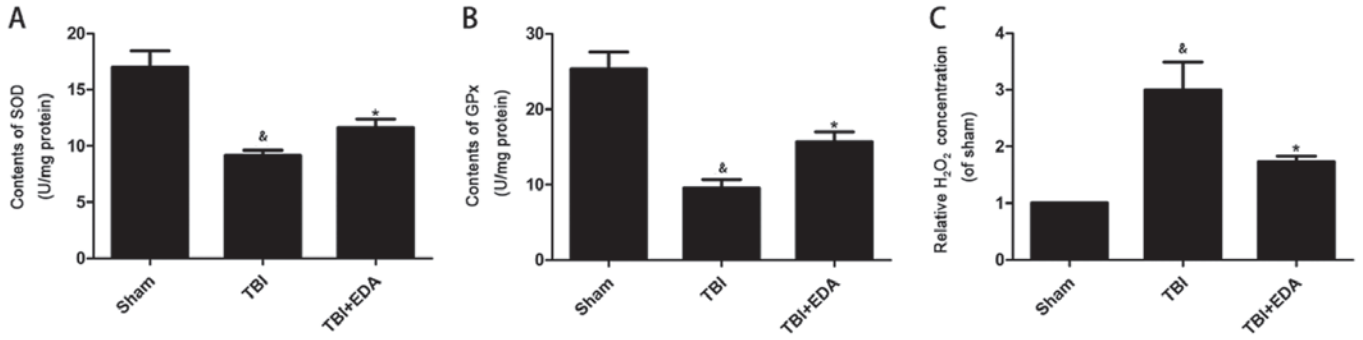

Figure 3. EDA treatment reduces the oxidative stress response following TBI. (A) SOD (B) GPx and (C) H_2O_2 levels in brain tissue (n=6). $^{\&}$P<0.05 vs. sham group; *P<0.05 vs. TBI group. EDA, edaravone; TBI, traumatic brain injury; SOD, superoxide dismutase; GPx, glutathione peroxidase; H_2O_2, hydrogen peroxide.

(Fig. 4G and H). EDA administration further elevated HO-1 and NQO-1 mRNA expression compared with the TBI group (Fig. 4G and H).

Discussion

TBI is a serious public health problem that can lead to permanent injury and neurological disorders (26). The associated secondary brain injury involves a series of pathological processes, including the inflammatory response (27) and oxidative stress (28), and is considered to be the leading cause of death and disability. Due to the lack of effective TBI treatments, new and effective therapies are urgently required. EDA is a novel free radical scavenger that exerts a number of beneficial biological functions, involving mechanisms that are anti-apoptotic (29), anti-inflammatory (23) and that alleviate stress on the endoplasmic reticulum (17). A previous study demonstrated that EDA can reduce oxidative stress, has anti-inflammatory effects and improves neurological deficits following concussion *in vivo* (30). The present results confirmed for the first time, to the best of our knowledge, that EDA treatment exerts a protective role in TBI by inhibiting the activation of NF-κB-mediated inflammation and promoting the Nrf2 antioxidant pathway.

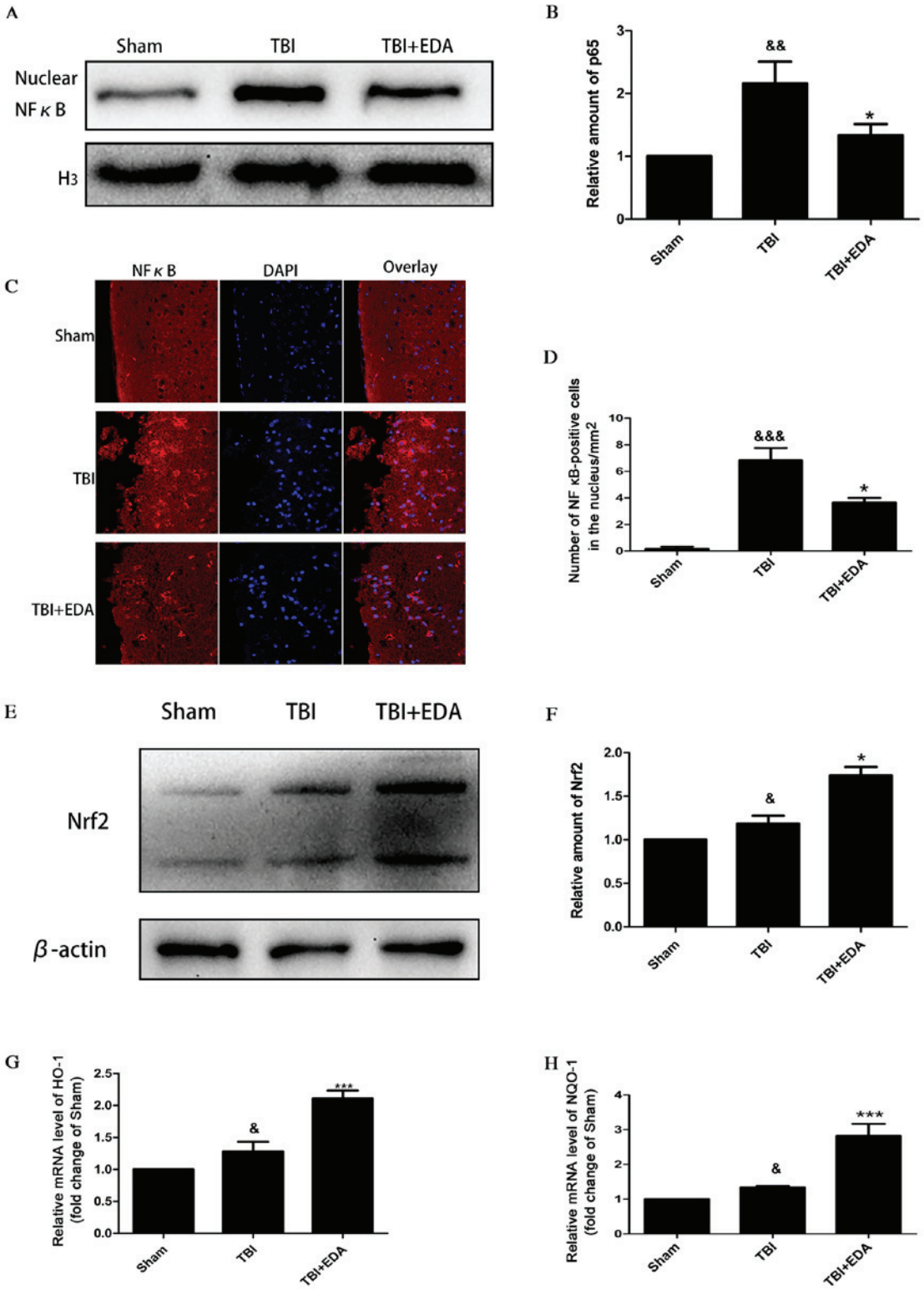

Figure 4. Effects of EDA on the NF-κB and Nrf2 signalling pathways following TBI. (A) The protein expression of NF-κB in the nucleus following treatment of TBI with EDA. (B) Optical density analysis of NF-κB protein. Protein levels were normalized to H3 and presented as ratio relative to the sham group. (C) Immunofluorescence staining (magnification, x400) of NF-κB (red) and DAPI (blue) in the various groups following TBI, with the merged immunofluorescent images demonstrating NFκB translocation from cytoplasm to nucleus. (D) Quantitative analysis of immunofluorescence data. (E) Nrf2 protein expression following treatment of TBI with EDA. (F) Optical density analysis of Nrf2 protein. Protein levels were normalized to β-actin and presented as ratio relative to the sham group. (G) NQO-1 and (H) HO-1 mRNA expression levels analyzed by reverse transcription-quantitative PCR (n=6). $^{\&}$P<0.05, $^{\&\&}$P<0.01 and $^{\&\&\&}$P<0.001 vs. sham group; *P<0.05 and ***P<0.001 vs. TBI group. EDA, edaravone; Nrf2, nuclear factor erythroid 2-related factor 2; TBI, traumatic brain injury.

NF-κB has a critical role in the inflammatory response induced by TBI, and previous research has demonstrated that EDA suppresses the systemic inflammatory response in a transient focal ischemia rat model (23). A study determined that EDA inhibits the activity of NF-κB induced by ischemia/reperfusion injury (19). In addition, EDA treatment

confers neuroprotective effects following TBI in rats (31). Based on the literature, the present study hypothesized that EDA may alleviate a TBI-induced inflammatory response by regulating the NF-κB protein. The present findings demonstrated that EDA treatment inhibited the translocation of NF-κB from the cytoplasm to the nucleus following TBI.

Oxidative stress occurs immediately following TBI. Recent studies have identified that EDA treatment regulates oxidative stress-related indicators *in vivo* and *in vitro* (9,20). It was also demonstrated that EDA attenuates hippocampal damage in an infant mouse model of pneumococcal meningitis via the Nrf2/HO-1 pathway (9). Therefore, the present study investigated the effect of EDA on these oxidative stress-related indicators. It was established that treatment with EDA reduced H_2O_2 level and elevated SOD and GPx activity in mice following TBI. Since EDA can activate the Nrf2/ARE pathway in the brains of mice, it was hypothesized that the protective effects of EDA following TBI may be mediated via the Nrf2/ARE signaling pathway. As predicted, the present results demonstrated that EDA exerts neuroprotective effects following TBI via the Nrf2/ARE signaling pathway.

The present study has limitations, for example, a key control group, such as TBI+vehicle, was missing in several experiments, which will be included in future work. However, numerous studies have demonstrated that vehicle administration alone does not affect inflammation and oxidative stress indicators, and has no protective effect on mice following TBI (32,33).

In conclusion, the present study demonstrated, for the first time, that EDA exerted neuroprotective effects following TBI, resulting in improved neurological function, attenuated neuronal apoptosis and inhibition of inflammation and oxidative stress. The beneficial effects of EDA treatment were achieved, at least partially, by promotion of Nrf2 expression and inhibition of NF-κB translocation into the nucleus. These findings provide evidence for the potential clinical application of EDA in TBI treatment.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MZ performed the experiments and drafted the manuscript. CHT performed analysis and interpretation of data. FFW acquired data and supervised the research. LYG revised the manuscript. JX and HYZ were responsible for acquisition of funding and supervision of the research group. DQC was responsible for the conception and design of the present study and financial support. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal study protocols were approved by the Animal Care and Use Committee of Wenzhou Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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