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Original Article

Asiatic acid attenuates traumatic brain injury via upregulating Nrf2 and HO-1 expression

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Abstract: The neuroprotective role of asiatic acid (AA) against cerebral ischemia has been previously reported. However, whether AA attenuates traumatic brain injury (TBI) has not been investigated. The current study aims to evaluate the effect of AA and its underlying mechanisms. We first established TBI model in SD rats. Neurological score, brain water content, and cell apoptosis were evaluated. Oxidative stress was detected by assessing the levels of malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE), and 8-hydroxy-2-deoxyguanosine (8-0HdG) in the brain tissue of rats 24 h after brain injury. Finally, we evaluated the expression of Nrf2 and HO-1 using Western blot and Real-time quantitative PCR. Our results suggest that AA improved neurological dysfunction, decreased brain edema and neuronal apoptosis. In addition, AA attenuated oxidative stress after TBI. Notably, AA up-regulated the expression levels of Nrf2 and HO-1. In summary, our study suggests that AA confers neuroprotection against TBI via reducing oxidative stress and enhancing Nrf2 and HO-1 expression.

Keywords: Asiatic acid, traumatic brain injury, Nrf2, HO-1

Introduction

Traumatic brain injury (TBI) is a leading cause of death and disability, particularly in young people. It occurs when a traumatic event causes the brain to move rapidly within the skull, leading to damage. Individuals of all ages, background, and health status are susceptible to TBI [1]. About 1.7 million people suffer TBI per year and TBI is listed as a contributing cause in about one third of injury-related deaths [2]. TBI is responsible for 50,000 deaths, leave 80,000 individuals with permanent disabilities and cost more than 77 billion dollars on average per year in the United States [3]. However, precise pathological mechanisms of TBI are lacking, the growing base of knowledge about TBI has put increased emphasis on its understanding and treatment.

TBI is understood to be the result of two phases: primary and secondary brain injury [1]. As the primary injury phase is unpredictable and immediate, it is not easily to treat. In the most severe cases, this phase of injury can be deadly. In the contrary, the delayed nature of secondary injury allows for multiple medical and surgical intervention and has become a major focus of TBI treatment. Many cellular processes are implicated in the secondary injury phase causing the death of neurons. These mechanisms include oxidative stress, ischemia, hypoxia, excitatory amino acids, inflammation and metabolic crisis. Oxidative stress plays an important role in TBI. Accumulating experiments have indicated the importance of regulator nuclear factor erythroid 2-related factor (Nrf2) in oxidative stress. Therefore, Nrf2/HO-1 pathways are widely studied in vertebrates. Nrf2 is a cytoprotective factor regulating the expression of genes coding for anti-oxidant including heme oxygenase-1 (HO-1) [4]. HO-1 is one of two distinct HO isoforms found in mammals which provide cytoprotective effect and play a crucial role in the development of oxidative [5]. The more detailed research of the regulation of Nrf2/HO-1 may provide new ideas for the treatment of TBI.

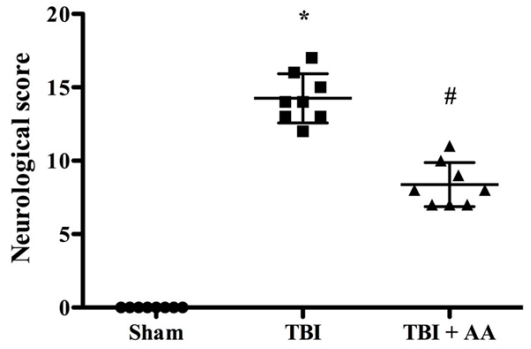

Figure 1. AA administration decreased mNSS 24 h after TBI. AA significantly attenuated neurological dysfunction. Compared with the TBI group, the neurological score in the TBI + AA group was significantly reduced ($P < 0.05$). The results were expressed as the mean ± SD, n = 6. $^{*}P < 0.05$ vs. the Sham group, $^{\#}P < 0.05$ vs. the TBI group.

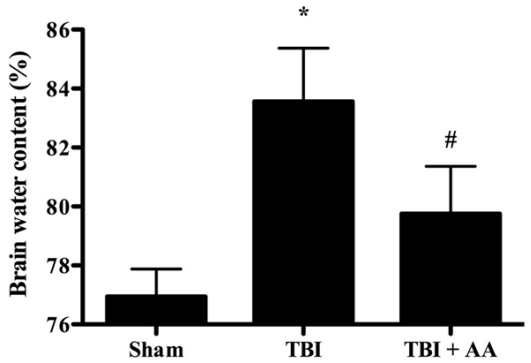

Figure 2. AA administration decreased brain water content 24 h after TBI. The results were expressed as the mean ± SD, n = 6. $^{*}P < 0.05$ vs. the Sham group, $^{\#}P < 0.05$ vs. the TBI group.

Asiatic acid (AA) is a pentacyclic triterpene obtained from the plant Centella asiatica which has been widely used as an anti-inflammatory and antioxidant agent [6]. It has shown to display robust protective properties in ischemic stroke [7], spinal cord injury [8], acute lung injury [9] and cardiac hypertrophy [10] through various pathways. However, the effects of AA on TBI and the precise mechanisms remain unclear. Therefore, this study aims to determine whether AA could alleviate TBI in rats as well as to reveal the potential mechanisms.

Materials and methods

Animals

Adult male Sprague-Dawley rats weighing 220-250 g were provided by the Experimental Animal Center of the Fourth Military Medical University. Animals were housed under controlled conditions on a 12 h light/dark cycle at 20°C and 60-70% humidity. All experimental procedures were approved by the Animal Use and Care Committee for Research of the Fourth Military Medical University, and were in accordance with the guidelines provided by the National Institute of Health (National Institutes of Health Publication No. 85-23, revised 1996).

Traumatic brain injury

TBI was induced by the controlled cortical impact injury model as previously reported [11]. All animals were anesthetized with 10% chloraldurat (0.5 g/kg, i.p.) and placed in the stereotaxic frame on a thermostatically-controlled heating pad to maintain body temperature. A midline incision was made to expose the skull, and left craniotomy with a diameter of 4.0 mm was made using a surgical electric cranial abrasive drill at 0.5 mm from the bregma. Rats were subjected to cortical impact injury at a 1.0 mm impact depth using an electromagnetic impactor (round tip: 3 mm diameter; speed: 4 m/s; dwell: 100 ms). For the sham group, the animals received the same anesthesia and surgical incision without the impact.

Animal groups and drug administration

The rats were randomly assigned into 3 experimental groups: (1) the Sham group (n = 30), (2) the TBI group (n = 30), (3) the TBI + AA group (n = 30). Asiatic acid (AA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). AA was solubilized with 1% dimethyl sulfoxide (DMSO) with 1% tween 20 in PBS. AA was administered intraperitoneally at a dose of 30 mg/kg 2 h and 6 h after TBI. The rats in the sham and TBI group were administered with the same volume of vehicle.

Evaluation of neurological deficit

Neurological deficits were evaluated 24 h after TBI by an observer who was blinded to experimental groups, using the modified neurological severity score (mNSS) [12]. The mNSS indicates motor, sensory, reflex and balance tests. The severity was as follows: 0: normal function; 1-6 points: mild injury; 7-12 points: moderate injury; and 13-18 points: severe injury. The maximum score of 18 points represents severe

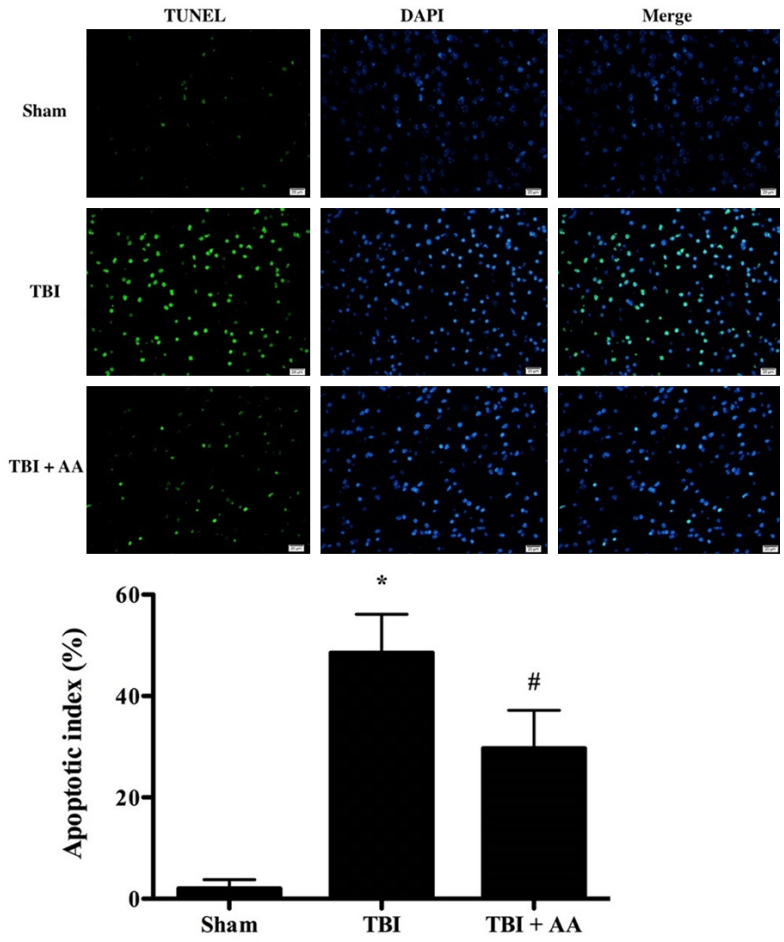

Figure 3. AA administration decreased neuronal apoptosis 24 h after TBI. The results were expressed as the mean ± SD, n = 6. $^{*}P < 0.05$ vs. the Sham group, $^{\#}P < 0.05$ vs. the TBI group.

neurological dysfunction, whereas 0 point indicates normal function.

Evaluation of brain water content

Rats were sacrificed after the behavioral tests by cervical dislocation, and brains were immediately removed and the ipsilateral cortical tissues were dissected and weighed to determine wet weight. After drying in an oven for 24 h at 110°C, weighed again to determine dry weight. Percentage of brain water content was calculated using the following formula: brain water content (%) = (wet weight-dry weight)/wet weight × 100%.

TUNEL staining

The apoptosis of neuron was assessed by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Briefly, brain sections were permeabilized with 1% proteinase K for 10 min, and 50 μL TUNEL reaction mixture was added on each section, and the sections were incubated in humidified atmosphere for 60 min at 37°C in the dark and then rinsed with PBS (pH 7.4) three times, for 5 min each time. Then the sections were incubated with DAPI for 5 min at room temperature in the dark, rinsed with PBS three times, and observed under a fluorescence microscope. The TUNEL-positive cells showed green fluorescence, and nuclei were stained with blue fluorescence. Apoptotic index was determined as the ratio of the number of TUNEL-positive neurons to the total number of neurons.

Detection of the levels of MDA, 4-HNE, and 8-OHdG in the brain tissue

The MDA level was detected using the MDA assay kit purchased from Sigma-Aldrich (St. Louis, MO, USA). The levels of 4-HNE and 8-OHdG were detected using the enzyme-linked immunosorbent assay (ELISA) kit according to the instructions of the manufacturers.

Western blot

The brain cortex protein from injured area was extracted using a lysis buffer with protease inhibitor. Equivalent amounts of protein were loaded and separated by 8-12% SDS-PAGE gel. After electrophoresis and transferred onto nitrocellulose membrane, the membranes were blocked in 5% skimmed milk, and then incubated with appropriate primary antibodies against Nrf2, HO-1, β-actin overnight at 4°C. Membranes were washed in TBST and incubated for 1 h at room temperature with the secondary antibodies. The protein bands were detected and

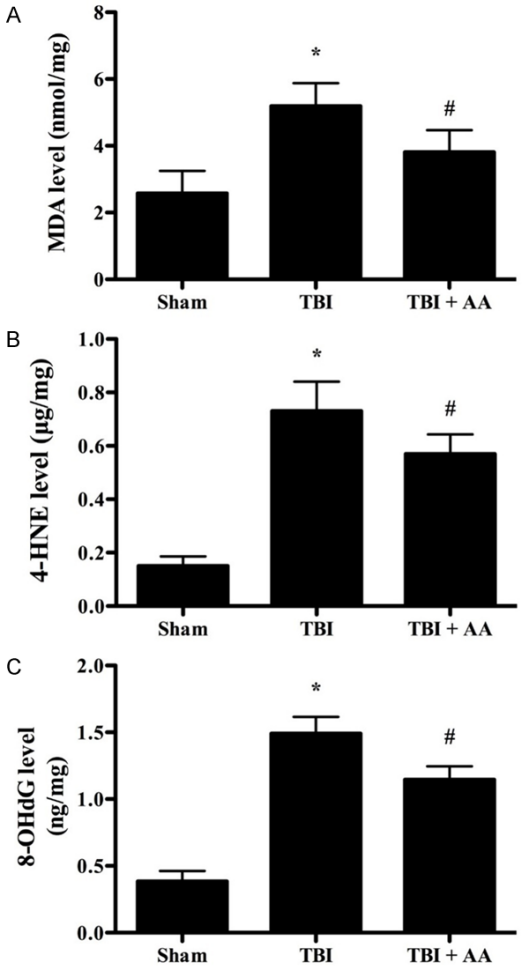

Figure 4. AA administration attenuated oxidative stress after TBI. A. MDA. B. 4-HNE. C. 8-OHdG. The results were expressed as the mean ± SD, n = 6. $^{*}P < 0.05$ vs. the Sham group, $^{\#}P < 0.05$ vs. the TBI group.

quantified by the Bio-Rad imaging system (Bio-Rad, Hercules, CA, USA).

Real-time quantitative PCR

Total mRNA was extracted by using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and was reverse-transcribed into cDNA according to the instructions. The Nrf2 upstream primer was 5'-GTTGAAGAACTCCTCCTGCTTG-3', and the downstream primer was 5'-GGCTTCCTC-ACTGGTATTGG-3'; the HO-1 upstream primer was 5'-GTCCCAGGATTTGTCCGAGG-3' and the downstream primer was 5'-GGAGGCCATCAC-CAGCTTAAA-3'. GAPDH upstream primer was 5'-AGTGTGACGTTGACATCCGTA-3' and the do-wn-stream primer was 5-GCCAGAGCAGTAA-TCTCCTTCT-3'. Each mRNA expression was normalized to GAPDH mRNA expression.

Statistical analysis

All data are presented as means ± SD and statistically analyzed by one-way ANOVA, followed by Bonferroni multiple comparisons test for intergroup comparisons. $P < 0.05$ was considered statistically significant.

Results

AA improved neurological deficits

As shown in **Figure 1**, mNSS dramatically increased 24 h after TBI, indicating neurological deficits after TBI. AA administration significantly reduced he neurological scores compared with the TBI group ($P < 0.05$). These data suggested that AA was beneficial for the improvement of neurological deficits after TBI.

AA reduced brain edema

As shown in **Figure 2**, the brain water content in the TBI group increased significantly compared with that in the Sham group. AA administration dramatically decreased the brain water content in the TBI + AA group compared with the TBI group ($P < 0.05$).

AA reduced neuronal apoptosis after TBI

TUNEL staining was used to detect the neuronal apoptosis after TBI. As shown in **Figure 3**, the apoptotic index in the Sham group was at a low level. TBI induced a marked increase in the number of TUNEL-positive cells. Compared with the TBI group, AA administration dramatically decreased the number of TUNEL-positive cells ($P < 0.05$).

AA reduced the levels of MDA, 4-HNE, and 8-OHdG

The levels of MDA, 4-HNE, and 8-OHdG were used to examine the oxidative stress after TBI (**Figure 4**). Compared with the Sham group, the levels of MDA, 4-HNE, and 8-OHdG significantly increased in the TBI group ($P < 0.05$). AA administration dramatically decreased the levels of MDA, 4-HNE, and 8-OHdG ($P < 0.05$).

AA enhanced the expression mRNA and protein levels of Nrf2 and HO-1

As Nrf2/HO-1 signaling is a critical signaling in attenuating oxidative stress, the effect of A on Nrf2/HO-1 signaling was evaluated. As shown

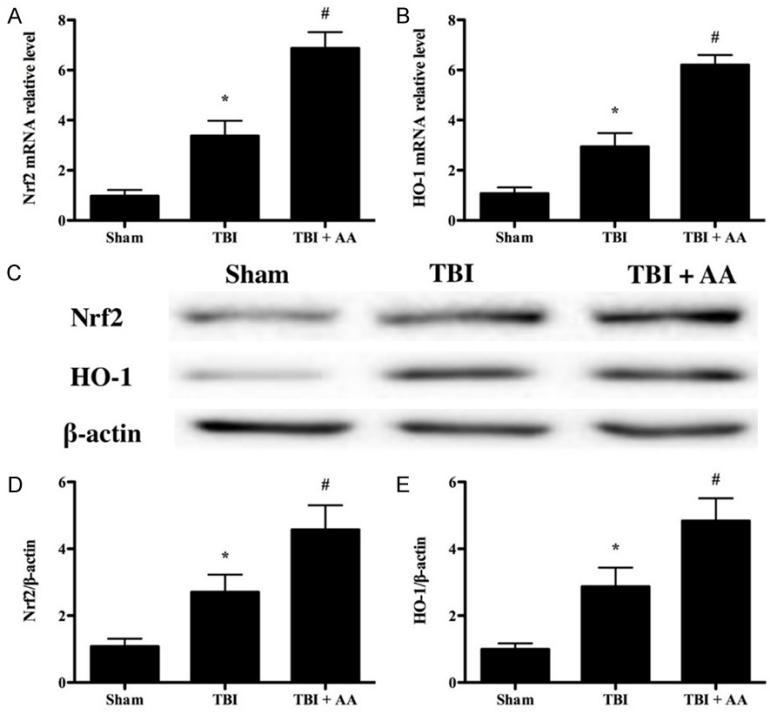

Figure 5. Effect of AA on the mRNA and protein expression of Nrf2 and HO-1 in the brain after TBI. A. Nrf2 mRNA level. B. HO-1 mRNA level. C. Nrf2 and HO-2 expression levels detected by Western blot. D. Nrf2/β-actin. E. HO-1/β-actin. The results were expressed as the mean ± SD, n = 6. $^{*}P < 0.05$ vs. the Sham group, $^{\#}P < 0.05$ vs. the TBI group.

in **Figure 5**, we first examined the mRNA levels of Nrf2 and HO-1. TBI induced a marked increase in the mRNA levels of Nrf2 and HO-1 ($P < 0.05$), which were further enhanced by AA administration ($P < 0.05$). The Western blot results showed that the protein levels of Nrf2 and HO-1 significantly increased after 24 h of TBI ($P < 0.05$). In comparison with the TBI group, the TBI + AA group exhibited further increased expressions of Nrf2 and HO-1 ($P < 0.05$).

Discussion

In the current study, we provided the evidence that AA administration attenuates neurological deficits and reduces brain edema induced by TBI in vivo and decreases neuronal apoptosis. Moreover, the increased levels of MDA, 4-HNE, and 8-OHdG following TBI was alleviated by AA administration. Notably, AA further enhanced the expression levels of Nrf2 and HO-1 after TBI.

We first induce CCI injury, which is a classical model in the study of TBI [11]. It has been reported that the mechanisms involved in the pathogenesis of TBI include neurological damage, brain edema, neuronal apoptosis, and oxidative stress [13, 14]. TBI can trigger primary and secondary injury in the brain, and secondary injury is the main target in the management of TBI.

The secondary injury of TBI is primarily due to oxidative stress [15]. Anti-oxidative stress is also an important therapeutic target in the management of TBI. Under normal circumstances, ROS level produced by metabolic activities are well managed by cellular antioxidant defense systems. When TBI occurs, the levels of ROS production overwhelm antioxidant defense systems and result in oxidative damage [16]. ROS and RNS are the main sources of oxidative stress in TBI. They are highly reactive because they attempt to gain electrons from surrounding substances, which can result in cell membrane, DNA and protein damage. MDA is an indicator of lipid peroxidation, which can occur in biological membranes (cell membrane and mitochondrial membrane), leading to the destruction and dysfunction of membranes. 4-HNE is also a product which can be generated in lipid peroxidation. 8-OHDG is a sensitive DNA damage marker and is produced following hydroxyl radical induction during oxidative stress, which can cause permanent alterations in DNA structure [17]. In our study, oxidative stress after TBI significantly increases as evidenced by the elevated levels of MDA, 4-HNE, and 8-OHdG. AA administration significantly decreases the levels of MDA, 4-HNE, and 8-OHdG. Our results indicate that AA attenuate oxidative stress via reducing free radicals. Moreover, neuronal apoptosis significantly increases after TBI, as evidenced by the increased number of TUNEL-positive cells. AA administration also reduces the number of TUNEL-positive cells after TBI. The result sug-

gests that AA administration can attenuate TBI via its anti-apoptotic effect.

Nrf2, a transcription factor of the endogenous defense system, can work against oxidative stress through the activation of its downstream targets, such as HO-1 [18]. Nrf2 is a basic-leucine zipper (bZIP) transcription factor that localizes in the cytoplasm and binds to Kelch-like ECH-associated protein 1 (Keap1), which is the inhibitor protein of Nrf2 [19]. After activated, Nrf2 disassociates from Keap1, translocates to the nucleus, and binds to the antioxidant response element (ARE) sequences in the promoter region of a group of cytoprotective genes and activates their transcription [20]. Therefore, Nrf2/ARE pathway is a therapeutic target for the treatment of TBI. It has been suggested that sinomenine confers neuroprotection against TBI via the Nrf2/ARE pathway [21]. In addition, Li et al. reported that quercetin protects against experimental TBI via the Nrf2 signaling pathway [22]. HO-1, as a critical downstream protein of Nrf2, is an antioxidant enzyme andcan be up-regulated in response to various oxidataive stimulations, such as UV light, hypoxia, and H_2O_2 [23]. In the present study, our data indicate that Nrf2 and HO-1 are found to be dramatically up-regulated after TBI at mRNA and protein levels. AA administration further increases the mRNA and protein levels of Nrf2 and HO-1. Our results indicate that the neuroprotective effect of AA is associated with the up-regulation of Nrf2 and HO-1.

In summary, our findings demonstrate that AA exhibits neuroprotection against TBI through activating the Nrf2/HO-1 pathway, an endogenous antioxidative system, indicating that AA appears to be a therapeutic candidate for the treatment of TBI.

Disclosure of conflict of interest

None.

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References

[1] Ghajar J. Traumatic brain injury. Lancet 2000; 356: 923-929.

[2] Summers CR, Ivins B and Schwab KA. Traumatic brain injury in the United States: an epidemiologic overview. Mt Sinai J Med 2009; 76: 105-110.

[3] Taylor CA, Bell JM, Breiding MJ and Xu L. Traumatic brain injury-related emergency department visits, hospitalizations, and deaths - United States, 2007 and 2013. MMWR Surveill Summ 2017; 66: 1-16.

[4] Alam J, Stewart D, Touchard C, Boinapally S, Choi AM and Cook JL. Nrf2, a Cap'n'collar transcription factor, regulates induction of the heme oxygenase-1 gene. J Biol Chem 1999; 274: 26071-26078.

[5] Wang B, Liu H, Yue L, Li X, Zhao L, Yang X, Wang X, Yang Y and Qu Y. Neuroprotective effects of pterostilbene against oxidative stress injury: involvement of nuclear factor erythroid 2-related factor 2 pathway. Brain Res 2016; 1643: 70-79.

[6] Hashim P, Sidek H, Helan MH, Sabery A, Palanisamy UD and Ilham M. Triterpene composition and bioactivities of centella asiatica. Molecules 2011; 16: 1310-1322.

[7] Lee KY, Bae ON, Serfozo K, Hejabian S, Moussa A, Reeves M, Rumbeiha W, Fitzgerald SD, Stein G, Baek SH, Goudreau J, Kassab M and Majid A. Asiatic acid attenuates infarct volume, mitochondrial dysfunction, and matrix metalloproteinase-9 induction after focal cerebral ischemia. Stroke 2012; 43: 1632-1638.

[8] Jiang W, Li M, He F, Bian Z, He Q, Wang X, Yao W and Zhu L. Neuroprotective effect of asiatic acid against spinal cord injury in rats. Life Sci 2016; 157: 45-51.

[9] Li Z, Xiao X and Yang M. Asiatic acid inhibits lipopolysaccharide-induced acute lung injury in mice. Inflammation 2016; 39: 1642-1648.

[10] Ma ZG, Dai J, Wei WY, Zhang WB, Xu SC, Liao HH, Yang Z and Tang QZ. Asiatic acid protects against cardiac hypertrophy through activating AMPKalpha signalling pathway. Int J Biol Sci 2016; 12: 861-871.

[11] Shen X, Li A, Zhang Y, Dong X, Shan T, Wu Y, Jia J and Hu Y. The effect of different intensities of treadmill exercise on cognitive function deficit following a severe controlled cortical impact in rats. Int J Mol Sci 2013; 14: 21598-21612.

[12] Chen J, Li Y, Wang L, Zhang Z, Lu D, Lu M and Chopp M. Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. Stroke 2001; 32: 1005-1011.

[13] Gao Y, Zhuang Z, Gao S, Li X, Zhang Z, Ye Z, Li L, Tang C, Zhou M, Han X and Li J. Tetrahydrocurcumin reduces oxidative stress-induced apoptosis via the mitochondrial apoptotic pathway by modulating autophagy in rats after

traumatic brain injury. Am J Transl Res 2017; 9: 887-899.
[14] Bruschetta G, Impellizzeri D, Campolo M, Casili G, Di Paola R, Paterniti I, Esposito E and Cuzzocrea S. FeTPPS reduces secondary damage and improves neurobehavioral functions after traumatic brain injury. Front Neurosci 2017; 11: 6.
[15] Abdul-Muneer PM, Chandra N and Haorah J. Interactions of oxidative stress and neurovascular inflammation in the pathogenesis of traumatic brain injury. Mol Neurobiol 2015; 51: 966-979.
[16] O'Connell KM and Littleton-Kearney MT. The role of free radicals in traumatic brain injury. Biol Res Nurs 2013; 15: 253-263.
[17] Witherell HL, Hiatt RA, Replogle M and Parsonnet J. Helicobacter pylori infection and urinary excretion of 8-hydroxy-2-deoxyguanosine, an oxidative DNA adduct. Cancer Epidemiol Biomarkers Prev 1998; 7: 91-96.
[18] Reichard JF, Motz GT and Puga A. Heme oxygenase-1 induction by NRF2 requires inactivation of the transcriptional repressor BACH1. Nucleic Acids Res 2007; 35: 7074-7086.
[19] Kaspar JW, Niture SK and Jaiswal AK. Nrf2:INrf2 (Keap1) signaling in oxidative stress. Free Radic Biol Med 2009; 47: 1304-1309.
[20] Kensler TW, Wakabayashi N and Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol 2007; 47: 89-116.
[21] Yang Y, Wang H, Li L, Li X, Wang Q, Ding H, Wang X, Ye Z, Wu L, Zhang X, Zhou M and Pan H. Sinomenine provides neuroprotection in model of traumatic brain injury via the Nrf2-ARE pathway. Front Neurosci 2016; 10: 580.
[22] Li X, Wang H, Gao Y, Li L, Tang C, Wen G, Zhou Y, Zhou M, Mao L and Fan Y. Protective effects of quercetin on mitochondrial biogenesis in experimental traumatic brain injury via the Nrf2 signaling pathway. PLoS One 2016; 11: e0164237.
[23] Cuadrado A and Rojo AI. Heme oxygenase-1 as a therapeutic target in neurodegenerative diseases and brain infections. Curr Pharm Des 2008; 14: 429-442.