Int J Clin Exp Med 2017;10(11):15213-15220
www.ijcem.com /ISSN:1940-5901/IJCEM0049459

Original Article

Emodin attenuates brain edema after traumatic brain injury in rats

Jian-Ping Zeng[1], Li-Tao Jia[2], Xin Jin[2], Ju-Ping Zheng[2], Xin-Wen Zhang[2], Ren-Ya Zhan[1]

[1]Department of Neurosurgery, The First Affiliated Hospital of Medical School of Zhejiang University, Hangzhou 310003, Zhejiang, P. R. China; [2]Department of Neurosurgery, Tongde Hospital of Zhejiang Province, Hangzhou 310012, Zhejiang, P. R. China

Received December 6, 2016; Accepted April 18, 2017; Epub November 15, 2017; Published November 30, 2017

Abstract: Background: Emodin, a major component extract of *Rhizoma Polygoni Cuspidati*, has various biological activities. The current study was to investigate the effects of emodin on brain edema after traumatic brain injury (TBI) in rats. Material and methods: Rat model of traumatic brain injury was established using Modified Feeney method, then rats were treated with emodin (10 mg/kg) 30 min before TBI and given once daily during the experiments. After neurological severity scores (NSS) evaluation at 1, 2 and 7 days, the animals were sacrificed. Brain samples were obtained and used for brain water content and blood-brain barrier (BBB) permeability measurement, as well as RT-PCR and Western blots. Results: Emodin significantly improved neurological function, reduced BBB permeability and ameliorated brain edema after TBI. It inhibited the expression of aquaporins (AQPs), including AQP-1, AQP-4 and AQP-9 both in mRNA and protein levels. Additionally, increased hypoxia-inducible factor-1α (HIF-1α) and matrix metalloprotein-9 (MMP-9) levels-induced by TBI were diminished after the treatment of emodin. Conclusion: Our results suggest that emodin exhibits neural protective effect on brain edema after TBI and the molecular mechanism is probably associated with the suppression of HIF-1α/AQPs and HIF-1α/MMP9 pathways.

Keywords: Brain edema, traumatic brain injury, emodin, blood-brain barrier, AQPs

Introduction

Traumatic brain injury (TBI), defined as direct mechanical damage to the brain, is a major cause of disability and death for people under 45 years old [1]. A major complication of TBI is brain edema due to the loss of blood brain barrier (BBB) permeability. Recent studies have suggested the overexpression of Aquaporins (AQPs) as a molecular mechanism for brain edema and destruction of BBB in TBI. AQPs are a family of water and water/glycerol channel proteins that responsible for the rapid transport of water across membranes [2]. AQP1 is expressed in the apical membrane of the choroid plexus, and plays a role in the formation of cerebrospinal fluid; AQP4, found in astrocyte foot processes, glia limitans and ependyma, facilitates water movement into and out of the brain, accelerates astrocyte migration and alters neuronal activity, while AQP9 is present in some glia and neuron, controls brain energy metabolism [3]. A recent research has demonstrated that AQP1 [4], AQP4 [5, 6] and AQP9 are involved in the brain edema formation and resolution. Furthermore, increased expression levels of the three proteins are also observed in the brain tissue after TBI [4, 6, 7]. Except for AQPs, Matrix metalloproteinase (MMP)-9 has been identified involving in the breakdown of BBB integrity and the formation of brain edema [8]. Additionally, increasing evidence indicates that hypoxia inducible factor (HIF)-1α expressed aberrantly after TBI, and is upstream regulator of AQPs and MMP-9 after TBI [6, 9, 10]. Therefore, inhibiting the HIF-1α/AQPs and HIF-1α/MMP9 pathways may be important targets for the treatment of TBI.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone), is a major component extract of dried rhizomes and the root of the *Rhizoma Polygoni Cuspidati*. It has been extensively studied due to its various biological activities [11], including

Table 1. Sequences of primers

Gene	Forward primer	Reverse primer
AQP1	5'-ATGAAGCCCAAATAGAGGA-3'	5'-CAGGAAACAGAAAGAAAGACA-3'
AQP4	5'-ATGCGGGTTTCTTGTTTC-3'	5'-TCAGATTTCGGCTCATAGTT-3'
AQP9	5'-GCATCTCTTTTCTCATCTCCT-3'	5'-GCCTTCACTTTATTCTCTGGT-3'
HIF-1α	5'-CCATTCCTCATCCATCAA-3'	5'-GCTCATAACCCATCAACTCA-3'
MMP-9	5'-GCAAGGATGGTCTACTGG-3'	5'-GAAGGTGAAGGGAAAGTGA-3'
GAPDH	5'-CTTTGGTATCGTGGAAGGACTC-3'	5'-GTAGAGGCAGGGATGATGTTCT-3'

anti-inflammatory [12], anti-oxidant [13], neuroprotective [14] and anti-cancer [15, 16] properties. Despite evidence has showed that emodin treatment can alleviate brain damage in a rat model of craniocerebral explosive injury by inhibiting inducible nitric oxide synthase (iNOS) [17], it is still unclear whether emodin exerts its protective effects on brain edema and BBB destruction in TBI. Interestingly, emodin has been reported to down-regulate the expression of AQPs [18, 19]. However, the relationship between emodin and AQPs has not been studied in TBI.

In this study, we aimed to investigate the role of emodin on the neurological deficits, brain edema, BBB permeability, and to primarily explore the underlying mechanism involved in.

Material and methods

Animal preparation and study design

All animal protocols were approved by University Committee on Animal Care and Use of Zhejiang University. A total of 36 adult male Sprague-Dawley rats (age, 6 to 8 weeks; weight, 200 to 250 g) were purchased from the Shanghai Laboratory Animals Co., Ltd. (Shanghai, China). All animals were randomly divided into three groups: Sham control, TBI rats orally treated with saline, TBI rats orally treated with emodin (10 mg/kg). The three groups were dived into 1, 2 and 7 day time-point sub-groups (each subgroup contained 4 rats). All of the experimental animals were maintained under a 12-hr light/dark cycle and were sacrificed for analysis on 1st, 2th, and 7th day after TBI injury.

TBI rat models

The TBI model was established using Modified Feeney method. Briefly, after anesthetization (xylazine/ketamine) and a midline skin incision, a 5-mm diameter craniotomy was created in the skull 5 mm behind the coronal suture and 3.5 mm beside the midline. After removing the skull, the right parietal cortex was exposed with the dura intact. Rats in TBI and Emodin groups were impacted following 20 g weight falling freely in the height of 30 cm to induce injury of the right parietal cortex. After impact, the craniotomy was closed with surgical staples and the rats were returned to their cage for recovery.

Assessment of neurological function

Neurological function were assessed on 1st, 2th, and 7th day after TBI using modified neurological severity scores (mNSS), a scoring range of 0-18, with higher scores reflecting greater extent of injury.

BBB permeability assessment

BBB permeability was detected by evaluat-ing Evans blue dye extravasation [20]. In brief, Evans blue dye (2%, 5 mL/kg) was injected intravenously and allowed to circulate for 1 h. The rats were perfused with PBS until clear PBS came out from the right atrium. The brains were removed and the left hemisphere tissue were then homogenized, centrifuged at 15,000×*g* for 30 min. Supernatant was collected and incubated with ethanol and trichloroacetic acid overnight, then centrifuged for 30 min at 15,000×*g*, the supernatant was used for spectrophotometric quantitation of extravasated Evans blue dye.

Brain water content measurement

Right hemisphere were removed and weighed immediately (wet weight). Then the tissues were weighed again after drying in an oven at 120°C for 24 h (dry weight). Brain water content was calculated as following: brain water content = ([wet weight - dry weight]/wet weight) 100%.

RT-PCR analysis

The mRNA expression levels of APQs, HIF-1α and MMP-9 were analyzed via PCR. Total RNA was extracted using TRIzol reagent (Gibco Life

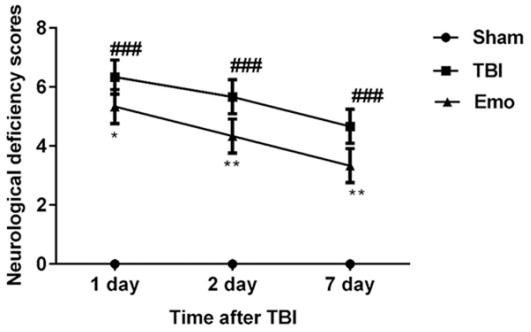

Figure 1. Effects of emodin in neurological function in rats after TBI. Data were showed as Mean ± SD, ###, P < 0.001, compared to Sham group; *, P < 0.05, **, P < 0.01, compared to TBI group.

Technologies; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The mRNA was then reverse-transcribed into cDNA using an M-MLV reverse transcription kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol with the ABI StepOnePlus Real-Time PCR system. Following the reverse transcription, fluorescence qPCR was performed using SYBR Green I dye (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. GAPDH was used as a normal control for mRNA analysis. The qRT-PCR data were exported and processed using the $^{\Delta\Delta}$Ct method. Primers were list at **Table 1**.

Western blotting

Equal amounts of each protein were processed for electrophoretic separation (SDS-PAGE) and subsequently electrotransfered to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The western blots were blocked with 5% milk at room temperature for 1 h. The membrane was incubated at 4°C overnight with primary antibody, followed by incubation for 1 h with a secondary anti-body conjugated with horseradish peroxidase. The immunoreactive bands were visualized using enhanced chemiluminescence (Cell Signaling Technology) and photographed by a chemiluminescence imaging system (ChemiDoc XRS+; Bio-Rad, Hercules, CA).

Statistical analysis

All data were expressed as Mean ± SD. Statistical analysis was performed by Graph Pad Prism (San Diego, CA). Comparing the difference between groups were statistically evaluated by one-way ANOVA with Student's test or Dunnett's test, neurological scores between different groups were statistically evaluated by two-way repeated measures ANOVA. P < 0.05 was considered as statistical significant.

Results

Emodin improves neurological function after TBI

To determine the effect of emodin on neurological function, the NSS were assessed on Days 1, 2 and 7 after TBI. All rats in Sham group received a score of 0, however, rats in TBI group showed obvious neural impairment (P < 0.001), and the NSS score was significantly increased in comparison to that of Sham group (1 day: P < 0.01; 2 days and 7 days: P < 0.001; **Figure 1**). What's important, obvious improvement was observed in the Emo group, compared with TBI group (P < 0.01).

Emodin reduced blood brain barrier permeability after TBI

Then, BBB permeability was measured among Sham, TBI, and Emo group rats on Day 1, 2, 7 by evaluating Evans blue dye extravasation. As shown in **Figure 2**, Evans blue dye extravasation in brain regions of rats in TBI group was significantly increased compared with Sham rats (P < 0.001). However, the treatment of emodin obviously reduced Evans blue dye extravasation in brain in comparison to rats in TBI group (1 day and 2 days: P < 0.05; 7 days: P < 0.001).

Emodin ameliorates brain edema after TBI

To determine whether emodin treatment attenuates brain edema after TBI, the water content among Sham, TBI, and Emo group rats on Day 1, 2, 7 was determined. As shown in **Figure 3**, the whole brain water content was significantly higher in TBI group (P < 0.001), compared to that in Sham group. However, emodin treatment greatly decreased brain water content (1 day and 2 days: P < 0.05; 7 days: P < 0.001).

Emodin down-regulated the expression of AQPs, HIF-1α and MMP-9 after TBI

To investigate the effect of emodin on AQPs, HIF-1α as well as MMP-9 after TBI, the mRNA

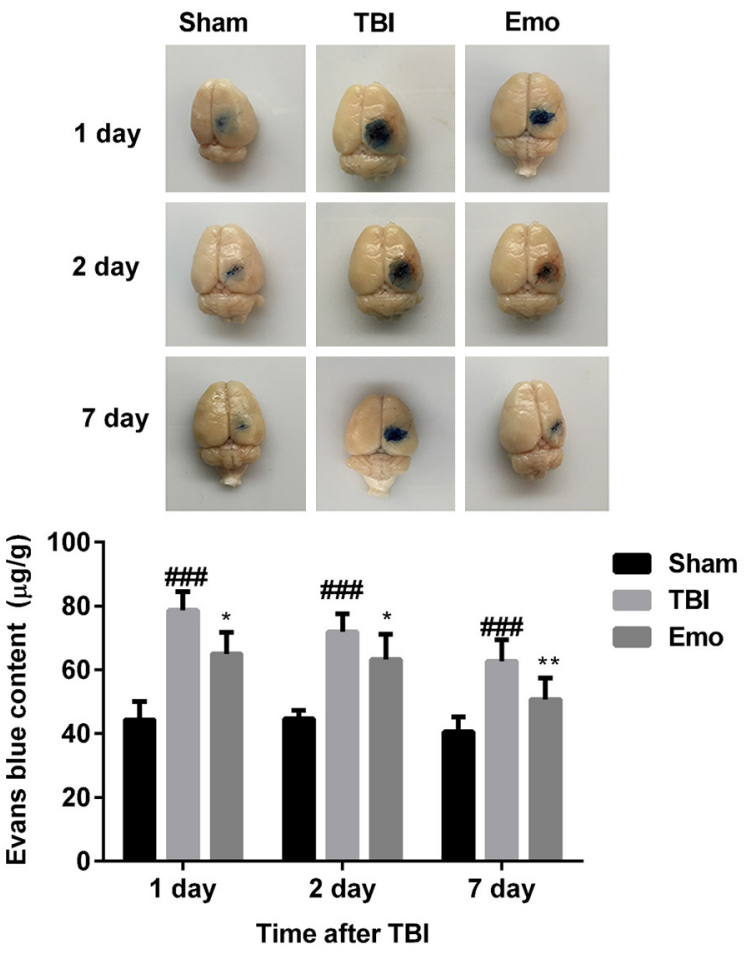

Figure 2. Effects of emodin in BBB permeability in rats after TBI. Data were showed as Mean ± SD, ###, P < 0.001, compared to Sham group; *, P < 0.05, **, P < 0.01, compared to TBI group.

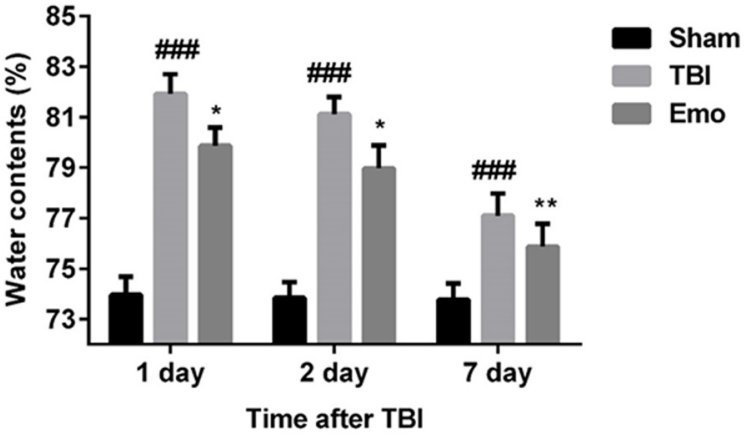

Figure 3. Effects of emodin in brain water contents in rats after TBI. Data were showed as Mean ± SD, ###, P < 0.001, compared to Sham group; *, P < 0.05, **, P < 0.01, compared to TBI group.

and protein expression of AQP1, AQP4 and AQP9 among Sham, TBI, and Emo group rats on Day 7 were detected with Q-PCR and Western Blot, respectively. As illustrated in **Figure 4A-C**, TBI surgery significantly increased the mRNA levels of AQP1, AQP4 and AQP9. However, emodin obviously reduced the elevation of mRNA levels of AQPs induced by TBI (**Figure 4A-C**). AQP1, AQP4 and AQP9 protein expression in TBI group, determined by Western blot, also significantly increased, similar to the mRNA expression (**Figure 4F**). After the treatment with emodin, protein expression levels of three AQPs were markedly down-regulated (**Figure 5**).

Lastly, the expression levels of HIF-1α and MMP-9 were measured among Sham, TBI and Emo group rats on Day 7. Increased mRNA expression of HIF-1α (**Figure 4D**) and MMP-9 (**Figure 4E**) were found in TBI group, while emodin treatment significantly reduced the mRNA expression of HIF-1α and MMP-9. In addition, the protein expression of HIF-1α and MMP-9 was inhibited in the Emo group on Day 7 after TBI as measured by Western blot (**Figure 5**).

Discussion

In this study, a TBI injury rat model was successfully established, as evidenced by significantly decreased neurological function, elevated blood brain barrier permeability and increased brain edema. Importantly, emodin treatment for 2 and 7 days greatly attenuated these changes above after TBI. To our knowledge, this work was the first study to indicate the pro-

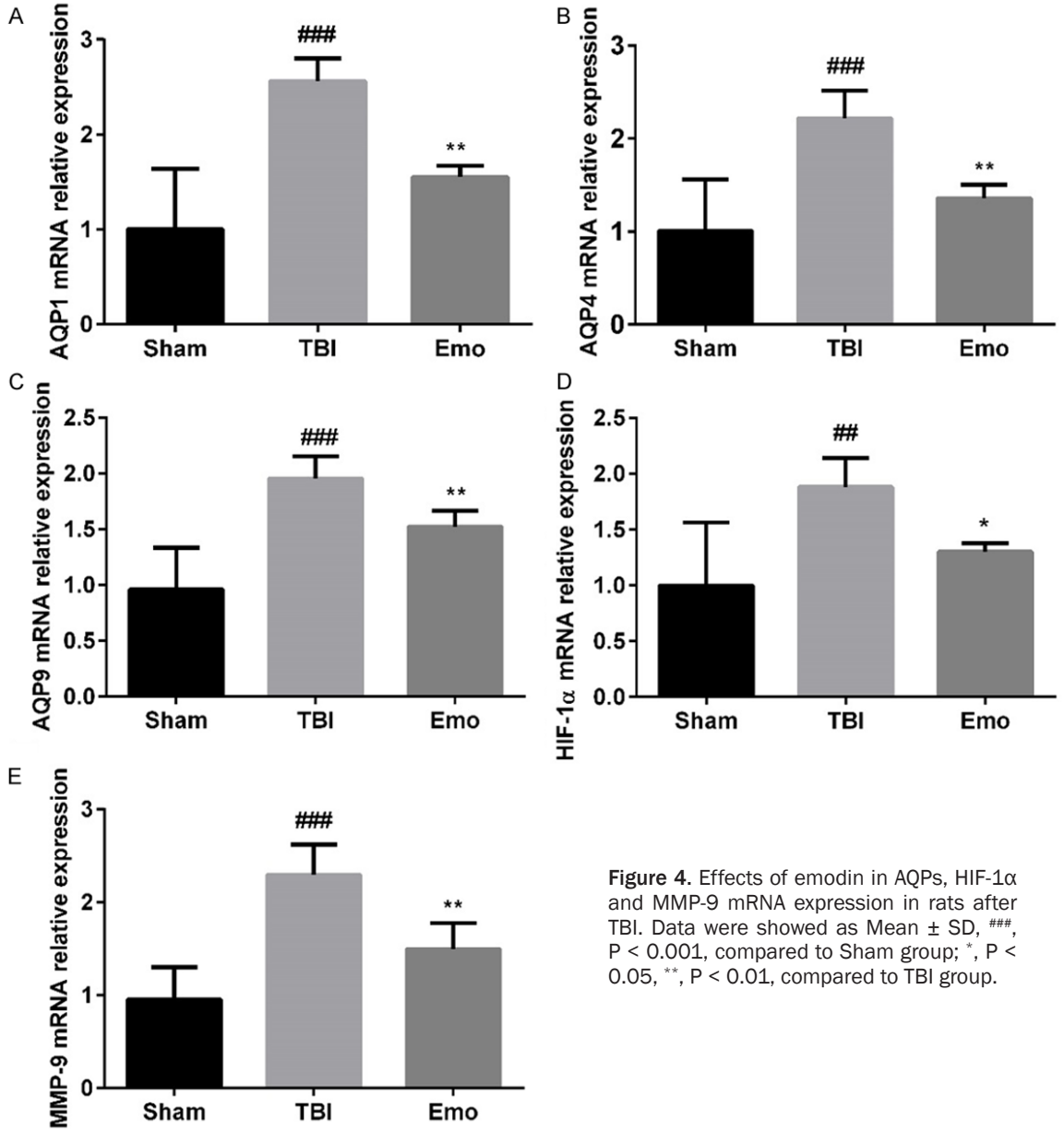

Figure 4. Effects of emodin in AQPs, HIF-1α and MMP-9 mRNA expression in rats after TBI. Data were showed as Mean ± SD, ###, $P < 0.001$, compared to Sham group; *, $P < 0.05$, **, $P < 0.01$, compared to TBI group.

tective effects of emodin treatment in rat model of TBI.

AQP1, 4 and 9 play key roles in the brain edema formation and resolution, and expressed aberrantly in various brain diseases in both rodent and human brain tissues [21-23]. AQP4 is the most abundant aquaporin in the brain, AQP4 deletion reduces brain edema after ischemic stroke [24] and pneumococcal meningitis [25]. AQP4 modulators have therapeutic potential in the treatment of brain edema in brain disorders including brain and spinal cord trauma injury [26]. According to these studies, the results in our study found that TBI surgery significantly increased the mRNA and protein levels of AQP4. However, treatment of emodin induced the decrease of AQP4 expression in rats after TBI. Similar results were also found in AQP1 and 9 expression after treatment of emodin. Consistently, the inhibitory effect of emodin in AQPs expression has been proven previously [18, 19]. Given the roles of AQP1 and 9 in the clearance of excess water and lactate and brain water homeostasis in TBI [27], the up-regulated expression of AQP1 and 9 by emodin may contribute to its protective effects in rats after TBI. Collectively, these findings above indicate that

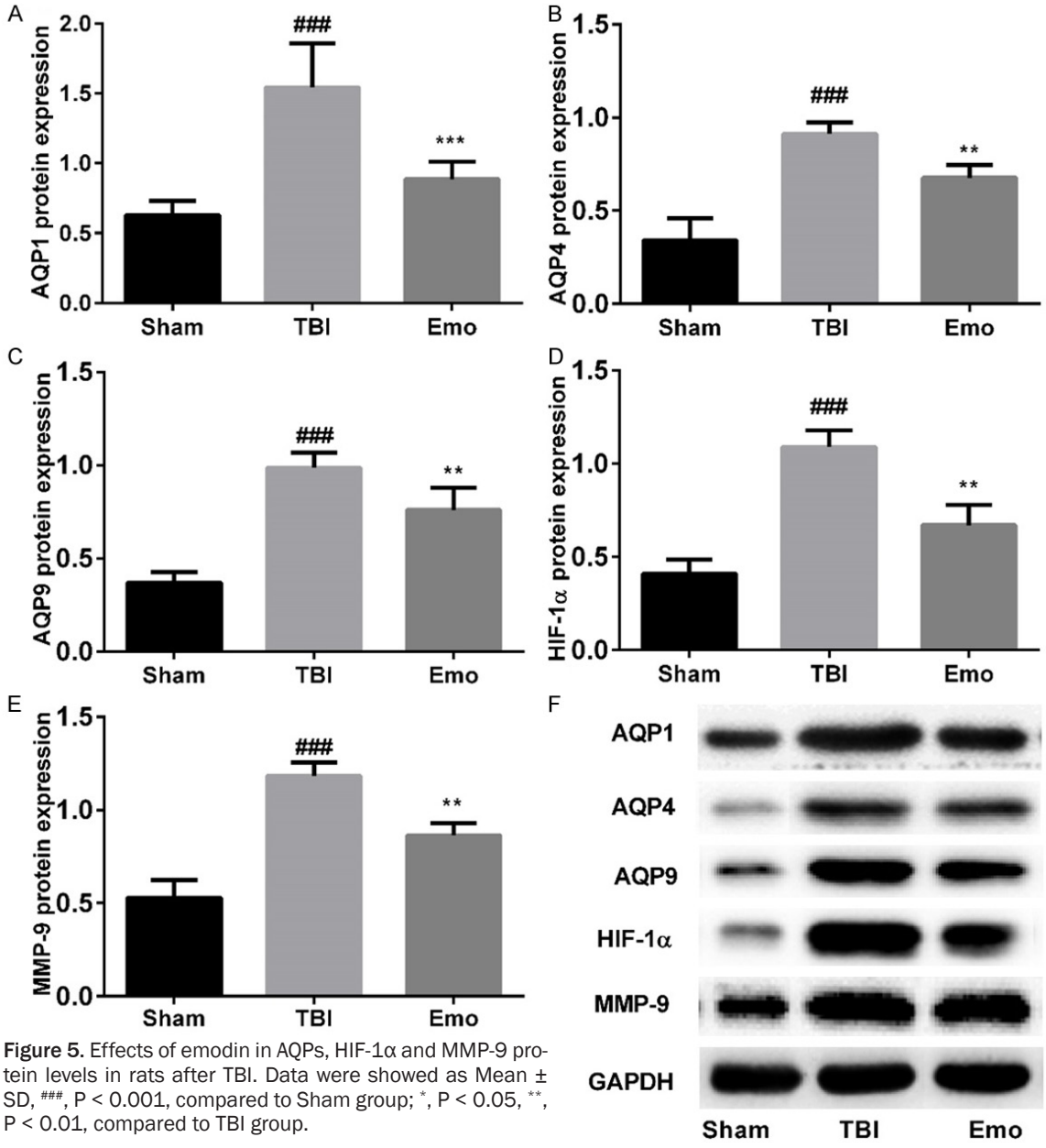

Figure 5. Effects of emodin in AQPs, HIF-1α and MMP-9 protein levels in rats after TBI. Data were showed as Mean ± SD, ###, $P < 0.001$, compared to Sham group; *, $P < 0.05$, **, $P < 0.01$, compared to TBI group.

the protective effects of emodin in TBI may be associated with the regulation of AQPs expression.

Furthermore, HIF-1α, a key transcription factor in response to physiological conditions, has been proven play a role in brain edema and BBB disruption TBI [6, 10]. TBI induces HIF-1α expression, which stimulates the expression of AQPs as well as MMP-9 [10]. Be agree with these studies, we found that HIF-1α and MMP-9 expression increased significantly after TBI. Additionally, Lu and colleagues [28] demonstrated that emodin could suppress HIF-1α and MMP-9 levels in human neuroblastoma cancer SH-SY5Y cells. Accordingly, our results showed that emodin treatment diminished the elevation expression of HIF-1α and MMP-9 in this animal model. Therefore, these results suggest that emodin attenuated brain edema and BBB dysfunction after TBI via the inhibition of HIF-1α, AQPs, and MMP-9.

Conclusion

Using a rat model we demonstrate that emodin treatment significantly attenuated brain injury after TBI, which is associated with significantly

reduced brain edema, attenuated BBB disruption by inhibiting HIF-1α/AQPs and HIF-1α/MMP-9 pathways. Thus, our data suggests emodin as a novel strategy for the treatment of TBI. However, further studies are still needed to further confirm and clarify the exact mechanism.

Acknowledgements

This work has been supported by grant of Traditional Chinese medicine science and technology plan of Zhejiang province (2A21316).

Disclosure of conflict of interest

None.

Address correspondence to: Ren-Ya Zhan, Department of Neurosurgery, The First Affiliated Hospital of Medical School of Zhejiang University, Hangzhou 310003, Zhejiang, P. R. China. E-mail: 1196057@zju.edu.cn

References

[1] Langlois JA, Rutland-Brown W and Wald MM. The epidemiology and impact of traumatic brain injury: a brief overview. J Head Trauma Rehabil 2006; 21: 375-378.

[2] Takata K, Matsuzaki T and Tajika Y. Aquaporins: water channel proteins of the cell membrane. Prog Histochem Cyto 2004; 39: 1-83.

[3] Tait MJ, Saadoun S, Bell BA and Papadopoulos MC. Water movements in the brain: role of aquaporins. Trends Neurosci 2008; 31: 37-43.

[4] Tran ND, Kim S, Vincent HK, Rodriguez A, Hinton DR, Bullock MR and Young HF. Aquaporin-1-mediated cerebral edema following traumatic brain injury: effects of acidosis and corticosteroid administration: Laboratory investigation. J Neurosurg 2010; 112: 1095-1104.

[5] Papadopoulos MC and Verkman AS. Aquaporin-4 and brain edema. Pediatr Nephrol 2007; 22: 778-784.

[6] Ding JY, Kreipke CW, Speirs SL, Schafer P, Schafer S and Rafols JA. Hypoxia-inducible factor-1α signaling in aquaporin upregulation after traumatic brain injury. Neurosci Lett 2009; 453: 68-72.

[7] Tourdias T, Mori N, Dragonu I, Cassagno N, Boiziau C, Aussudre J, Brochet B, Moonen C, Petry KG and Dousset V. Differential aquaporin 4 expression during edema build-up and resolution phases of brain inflammation. J Neuroinflammation 2011; 8: 143.

[8] Alluri H, Wilson RL, Anasooya Shaji C, Wiggins-Dohlvik K, Patel S, Liu Y, Peng X, Beeram MR, Davis ML, Huang JH and Tharakan B. Melatonin preserves blood-brain barrier integrity and permeability via matrix metalloproteinase-9 inhibition. PLoS One 2016; 11: e0154427.

[9] Zhang J, Xiong Y, Lu LX, Wang H, Zhang YF, Fang F, Song YL and Jiang H. AQP1 expression alterations affect morphology and water transport in Schwann cells and hypoxia-induced upregulation of AQP1 occurs in a HIF-1alpha-dependent manner. Neuroscience 2013; 252: 68-79.

[10] Higashida T, Kreipke CW, Rafols JA, Peng C, Schafer S, Schafer P, Ding JY, Dornbos III D, Li X and Guthikonda M. The role of hypoxia-inducible factor-1α, aquaporin-4, and matrix metalloproteinase-9 in blood-brain barrier disruption and brain edema after traumatic brain injury: laboratory investigation. J Neurosurg 2011; 114: 92-101.

[11] Dong X, Fu J, Yin X, Cao S, Li X, Lin L and Ni J. Emodin: a review of its pharmacology, toxicity and pharmacokinetics. Phytother Res 2016; 30: 1207-1218.

[12] Han JW, Shim DW, Shin WY, Heo KH, Kwak SB, Sim EJ, Jeong JH, Kang TB and Lee KH. Anti-inflammatory effect of emodin via attenuation of NLRP3 inflammasome activation. Int J Mol Sci 2015; 16: 8102-8109.

[13] Ahn SM, Kim HN, Kim YR, Choi YW, Kim CM, Shin HK and Choi BT. Emodin from polygonum multiflorum ameliorates oxidative toxicity in HT22 cells and deficits in photothrombotic ischemia. J Ethnopharmacol 2016; 188: 13-20.

[14] Liu T, Jin H, Sun QR, Xu JH and Hu HT. Neuroprotective effects of emodin in rat cortical neurons against β-amyloid-induced neurotoxicity. Brain Res 2010; 1347: 149-160.

[15] Zu C, Zhang M, Xue H, Cai X, Zhao L, He A, Qin G, Yang C and Zheng X. Emodin induces apoptosis of human breast cancer cells by modulating the expression of apoptosis-related genes. Oncol Lett 2015; 10: 2919-2924.

[16] He Y, Huang J, Wang P, Shen X, Li S, Yang L, Liu W, Suksamrarn A, Zhang G and Wang F. Emodin potentiates the antiproliferative effect of interferon α/β by activation of JAK/STAT pathway signaling through inhibition of the 26S proteasome. Oncotarget 2016; 7: 4664.

[17] Ma Y, Xia X, Cheng JM, Kuang YQ, Yang T, Yang LB, Fan K and Gu JW. Emodin inhibits inducible nitric oxide synthase in a rat model of craniocerebral explosive injury. Neurochem Res 2014; 39: 1809-1816.

[18] Zheng YF, Liu CF, Lai WF, Xiang Q, Li ZF, Wang H and Lin N. The laxative effect of emodin is attributable to increased aquaporin 3 expression in the colon of mice and HT-29 cells. Fitoterapia 2014; 96: 25-32.

[19] Yanni S, Lijing S, Shuqing L, Juan S, Jiejing C and Jun L. Effect of emodin on aquaporin 5 expression in rats with sepsis-induced acute lung injury. J Tradit Chin Med 2015; 35: 679-684.
[20] Cao S, Zhu P, Yu X, Chen J, Li J, Yan F, Wang L, Yu J and Chen G. Hydrogen sulfide attenuates brain edema in early brain injury after subarachnoid hemorrhage in rats: possible involvement of MMP-9 induced blood-brain barrier disruption and AQP4 expression. Neurosci Lett 2016; 621: 88-97.
[21] Fukuda AM, Pop V, Spagnoli D, Ashwal S, Obenaus A and Badaut J. Delayed increase of astrocytic aquaporin 4 after juvenile traumatic brain injury: possible role in edema resolution? Neuroscience 2012; 222: 366-378.
[22] Badaut J, Hirt L, Granziera C, Bogousslavsky J, Magistretti PJ and Regli L. Astrocyte-specific expression of aquaporin-9 in mouse brain is increased after transient focal cerebral ischemia. J Cereb Blood Flow Metab 2001; 21: 477-482.
[23] Badaut J, Brunet JF, Grollimund L, Hamou MF, Magistretti PJ, Villemure JG and Regli L. Aquaporin 1 and aquaporin 4 expression in human brain after subarachnoid hemorrhage and in peritumoral tissue. Acta Neurochir Suppl 2003; 86: 495-498.
[24] Manley GT, Fujimura M, Ma T, Noshita N, Filiz F, Bollen AW, Chan P and Verkman AS. Aquaporin-4 deletion in mice reduces brain edema after acute water intoxication and ischemic stroke. Nat Med 2000; 6: 159-163.
[25] Papadopoulos MC and Verkman AS. Aquaporin-4 gene disruption in mice reduces brain swelling and mortality in pneumococcal meningitis. J Biol Chem 2005; 280: 13906-13912.
[26] Papadopoulos MC and Verkman AS. Potential utility of aquaporin modulators for therapy of brain disorders. Prog Brain Res 2008; 170: 589-601.
[27] Liu H, Yang M, Qiu GP, Zhuo F, Yu WH, Sun SQ and Xiu Y. Aquaporin 9 in rat brain after severe traumatic brain injury. Arq Neuropsiquiatr 2012; 70: 214-220.
[28] Lu HF, Lai KC, Hsu SC, Lin HJ, Kuo CL, Liao CL, Yang JS and Chung JG. Involvement of matrix metalloproteinases on the inhibition of cells invasion and migration by emodin in human neuroblastoma SH-SY5Y cells. Neurochem Res 2009; 34: 1575-1583.