

Original Research Article

Asiatic acid exerts neuroprotective effect against hypoxic-ischemic brain injury in neonatal rats via inhibition of oxidative damage

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Abstract

Purpose: To investigate the effect of asiatic acid on hypoxic ischemia-induced injury in neonatal rats, and the underlying mechanism of action.

Methods: Hypoxic-ischemia (HI) neonatal rat model was established via permanent ligation of the carotid artery, followed by hypoxia (exposure to 8 % oxygen and 92 % nitrogen) for 24 h. Immunofluorescence, using fluorescence microscope, was used for the determination of expressions of p-TAK1, NeuN and GFAP. Western blotting was used for assaying protein expression levels, while TUNEL assay was employed for the measurement of apoptosis.

Results: Treatment of rats with asiatic acid prior to HI effectively prevented up-regulation of pTAK1 and decreased the count of p-TAK1-containing astrocytes. The proportion of NeuN containing p-TAK1 in HI rat brain cortex was significantly reduced by asiatic acid ($p < 0.05$). Treatment of rats with asiatic acid suppressed HI-induced up-regulation of pJNK expression. The HI-induced increase in the expression levels of caspase-3, p53 and p-c-Jun in rat brain cortex were reversed by asiatic acid ($p < 0.05$). The HI-mediated up-regulation of expressions of p-JNK, caspase-3, p53 and p-c-Jun in rat brain cortex were inhibited significantly by NG25. Asiatic acid treatment also significantly alleviated HI-mediated increase in apoptosis of neurons in rat brain cortex, when compared to model group ($p < 0.05$).

Conclusion: These findings suggest that asiatic acid prevents HI-induced brain injury in neonatal rats via inhibition of neuronal apoptosis. Moreover, it inhibits TAK1 activation, suppresses p-JNK expression and targets pro-apoptotic factors in brain cortex. Therefore, asiatic acid may be a therapeutic agent for the management of HI-induced brain injury.

Keywords: Hypoxic-ischemic, Neuroprotection, Epilepsy, Therapeutic, Apoptosis

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INTRODUCTION

Hypoxia-ischemia (HI) injury has been reported in about 6 out of 1,000 births worldwide, leading to chronic disability and high mortality [1]. In

humans, HI results in perinatal injury to brain which ultimately causes seizures, impaired learning, cerebral palsy and epilepsy [2]. It has been reported that the mechanism involved in neonatal brain injury is different from that

associated with brain injury in adults [3]. Induction of HI activates apoptosis in neonatal rats, thereby causing injury and impairing brain development [4]. Therefore, brain injury associated with HI induction may be effectively prevented through targeting of apoptotic pathways.

Mitogen-activated protein kinase (MAPK), a member of TGF- β -activated kinase-1 (TAK1) family is activated by morphologic proteins of the bones and TGF- β [5]. Under the influence of some stressors, the activated TAK1 plays crucial role in the up-regulation of I κ B kinase and nuclear factor- κ B pathways [6]. Moreover, c-Jun N-terminal kinase (JNK) and p38 MAPK stress factor-associated pathways are activated by TAK1 [6]. It has been reported that TAK1 plays a major role in cell death regulation after activation by different stimuli [7]. In pulmonary and thyroid carcinoma cells, TAK1-JNK pathway has been found be vital in activation of apoptosis [8,9]. Studies have demonstrated that targeting of TAK1-JNK pathway may be of vital therapeutic significance for different kinds of disorders and cancers [10]. Asiatic acid (Figure 1) is a compound that belongs to triterpenoid family consisting of five rings fused together, and it is present in *Centella asiatica* [11]. The compound exhibits neuroprotective properties *in vitro* and *in vivo* in animal models [11]. It has been reported that asiatic acid protected B103 neuroblastoma cells from apoptosis induced by β -amyloid and H₂O₂ exposure [12]. Moreover, glutamate-mediated toxicity on rat cortical cells was prevented by asiatic acid through activation of cellular antioxidant defence [13]. The present study was carried out to investigate the effect of asiatic acid on hypoxia-ischemia-induced injury in neonatal rats, as well as the underlying mechanism.

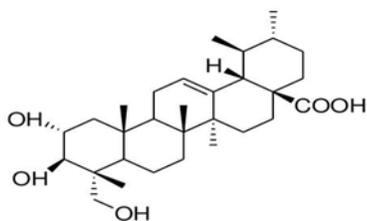


Figure 1: Chemical structure of asiatic acid

EXPERIMENTAL

Establishment of rat model of HI neonatal

A total of 50 neonatal rats (mean weight = 22 g) were obtained from the animal house of Sichuan University, China. The neonatal rats were maintained at 23 ± 2 °C in an environment with

12-h light/12-h dark cycle, and were given free access to feed and water. Hypoxia-ischemia (HI) was induced in the neonatal rats under diethylether anesthesia (0.1 mg/kg). After administration of the anesthesia, the rats were kept on homiothermy bench maintained at a temperature at 37 °C. The common carotid artery on right side was carefully exposed by making an incision through the midline of the neck. Then, 5-0 silk thread was used for permanent ligation of the artery, and the rats were allowed to recover from anesthesia. After 30 min, the pups were kept in hypoxia chambers (8 % oxygen and 92 % nitrogen) at a constant temperature of 37 °C for 24 h. Pups in the sham group were given incision, and 5-0 silk was kept around carotid artery, but ligation was not performed. Asiatic acid was intragastrically administered to the pups at doses of 2, 4 and 10 mg/kg 1 h before HI induction. The rats received NG25 in the right cerebral hemisphere, 30 min. before HI induction. The experimental methodology used in this study was in accordance with National Institute of Health guidelines [14]. The study was approved by Animal Ethics Committee of Northwest Women and Children's Hospital, Shaanxi, China (approval number = NWCH/0017/076).

Immunofluorescence

At 24 h after HI induction, the rat pups were anesthetized using ketamine injection at a dose of 100 mg/kg, along with xylazine (10 mg/kg) *via* intraperitoneal route. The pups were sacrificed by decapitation and the brain samples were excised, perfused and subsequently fixed for 48 h in 4 % paraformaldehyde. The brains were embedded in paraffin and cut into 3-mm thin sections using a microtome. Then, the sections were probed for protein expression by incubating them at 4 °C with primary antibodies against p-TAK1 (CAS no. 9339, dilution 1:200); NeuN (CAS no. MAB377, dilution 1:150), and GFAP (CAS no. MAB360, dilution 1:80). Thereafter, the sections were incubated with fluorescein isothiocyanate-conjugated secondary antibody (CAS no. sc-2012) at 37 °C. The cell nuclei were dyed with 4',6-diamidino-2-phenylindole, and the sections were randomly imaged at five fields using a fluorescence microscope (DTX500; Nikon Corporation, Tokyo, Japan).

Western blot analysis

At 24 h after HI induction, the rat pups were anesthetized with ketamine injection (100 mg/kg) and xylazine (10 mg/kg) *via* intraperitoneal route. The brain samples were excised, and the cortex

and hippocampus were isolated from the right hemisphere. The tissues were washed with PBS and homogenized with RIPA lysis buffer (Haimen, China) for 25 min. The homogenate was centrifuged for 15 min at 10,000 rpm, and the protein content of the supernatant was measured using BCA protein kit (Beyotime Institute of Biotechnology). Then, 20- μ g protein samples were resolved using 10 % SDS-PAGE and subsequently transferred onto PVDF membranes. The membranes were blocked by incubation with 5 % non-fat milk and TBS containing 0.05 % Tween-20 for 1 h. Thereafter, the membrane was incubated overnight at 4 °C with primary antibodies against p-TAK1 (cat no. 9339, 1:1,000); TAK1 (cat no. 5206, 1:800); p-JNK (cat no. 9255, 1:1,000); p-c-Jun (cat no. 3270, 1:800, all from Cell Signaling Technology, Inc.); p53 (cat no. ab26, 1:200; Abcam), and caspase-3 (cat no. C9598, 1:100). Then, the membrane was washed with PBS and incubated for 2 h with horseradish peroxidase-conjugated secondary antibody at room temperature. Visualization of bands was carried out with electrochemiluminescence detection kit (Merck KGaA), while quantification was done with Image-Pro Plus (version 6.0; Inc, Rockville, MD, USA).

TUNEL assay

TUNEL assay was employed for determination of apoptosis in brain cortex tissues of the neonatal rats at 24 h after HI induction, using DeadEnd™ Fluorometric system (Promega Corporation, Madison, WI, USA). Cellular apoptotic nuclei were identified using dark-green fluorescence staining of the tissues, while a fluorescence microscope was used for quantification of apoptotic cells in five randomly selected regions of the tissues.

Statistical analysis

Data are presented as mean \pm SD of triplicate measurements. The data were analyzed using SPSS version 19.0 (SPSS, Inc., Chicago, IL, USA). Differences amongst groups were determined using One-Way Analysis of Variance (ANOVA) and Tukey's post hoc test. The differences were assumed statistically significant at $p < 0.05$.

RESULTS

Asiatic acid inhibited TAK1 phosphorylation in neonatal rats with HI

There was a significant increase in expression of p-TAK1 in rat brain cortex at 24 h after HI,

when compared with the sham group (Figure 2). However, treatment of the rats with asiatic acid prior to HI induction effectively and dose-dependently prevented up-regulation of p-TAK1 in brain cortex, relative to model group. Treatment of rats with asiatic acid at doses of 2 and 4 mg/kg significantly suppressed HI-induced p-TAK1 expression in brain cortex. In HI rats, asiatic acid treatment at a dose of 10 mg/kg suppressed p-TAK1 expression, relative to the sham group.

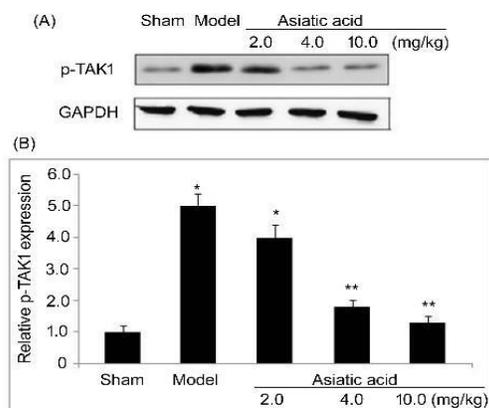


Figure 2: Effect of asiatic acid on TAK1 expression in neonatal rats. (A) TAK1 expression in brain cortex HI rats pre-treated with asiatic acid at doses of 2, 4 and 10 mg/kg, as measured using western blotting. (B) Quantified TAK1 expression at 24 h after HI induction in brain cortex, with GAPDH as control; ** $p < 0.018$; ** $p < 0.09$ vs. sham

Asiatic acid suppressed p-TAK1-containing GFAP cell count in HI rat brain cortex

The HI model rats had markedly higher proportion of astrocytes containing p-TAK1 in the brain cortex than the sham group (Figure 3). The count of astrocytes containing p-TAK1 in the HI rat brain cortex was significantly decreased on treatment with asiatic acid at a dose of 10 mg/kg.

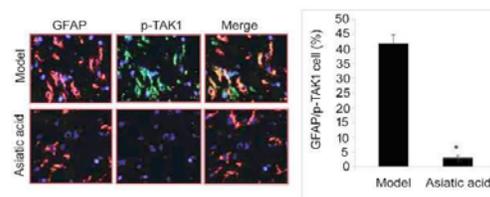


Figure 3: Effect of asiatic acid on GFAP staining in HI rat brain cortex. The HI rats were pre-treated with asiatic acid at doses of 2, 4 and 10 mg/kg. The tissues were subjected to double immunofluorescence GFAP (red) + p-TAK1 (green) staining. The nuclei of the cells were stained with DAPI

Asiatic acid suppressed count of p-TAK1-containing NeuN cells in HI rat brain cortex

Immunofluorescence data revealed significantly higher count of NeuN cells containing p-TAK1 in HI rat brain cortex than in the sham group (Figure 4). The proportion of NeuN cells containing p-TAK1 was markedly reduced in HI rat brain cortex on treatment with asiatic acid at a dose of 10 mg/kg.

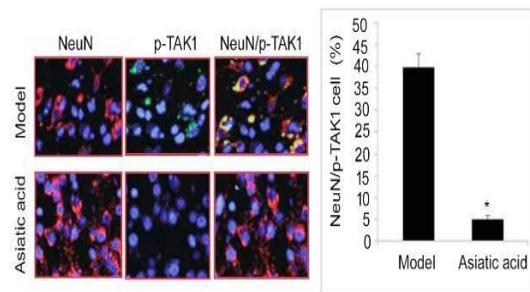


Figure 4: Asiatic acid suppressed p-JNK expression in HI rat brain cortex. The HI rats were pre-treated with asiatic acid at doses of 2, 4 and 10 mg/kg. The tissue sections embedded in paraffin were subjected to double immunofluorescence NeuN (red) + p-TAK1 (green) at 24 h after HI induction

The HI induction significantly promoted p-JNK expression in brain cortex of the rats at 24 h, when compared to sham group (Figure 5). However, asiatic acid treatment markedly reduced HI-induced up-regulation of p-JNK expression in a dose-based manner. The HI-induced increase in p-JNK expression in rat brain cortex was reversed completely on treatment with asiatic acid at a dose of 10 mg/kg. Moreover, marked elevation in levels of caspase-3, p53 and p-c-Jun were observed in the brain cortex of rats following HI. However, the HI-induced increases in expression levels of caspase-3, p53 and p-c-Jun in rat brain cortex were reversed. by asiatic acid treatment.

Effect of NG25 on expression of p-JNK in HI rat brain cortex

Administration of NG25 (p-TAK1 inhibitor) caused a significant reduction in p-TAK1 expression in the brain cortex of HI rats (Figure 6). The HI-mediated up-regulation of p-JNK expression was also suppressed in rat brain cortex by administration of NG25. Moreover, the HI-induced up-regulations in expression levels of caspase-3, p53 and p-c-Jun in rat brain cortex were markedly reversed on administration of NG25.

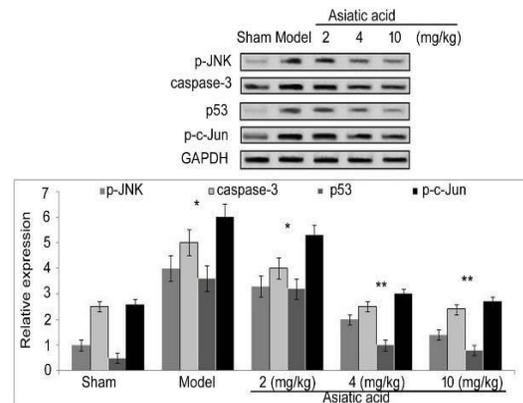


Figure 5: Effect of asiatic acid on JNK activation in HI rats. (A) Expression levels of p-JNK, caspase-3, p53 and p-c-Jun in brain cortex of rats pre-treated with asiatic acid at doses of 2, 4 and 10 mg/kg prior to HI, as measured using western blotting. (B) Quantification of protein expression levels in rat brain cortex at 24 h after HI induction, with GAPDH as control. ** $p < 0.018$; ** $p < 0.09$ vs. sham

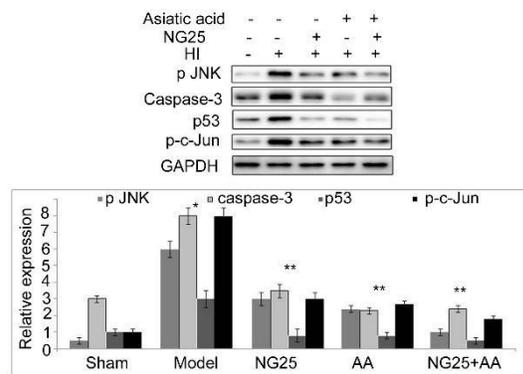


Figure 6: Effect of NG25 administration on p-JNK expression. (A) Expression levels of p-JNK, caspase-3, p53 and p-c-Jun in brain cortex of HI rats administered NG25, as measured using western blotting. (B) Quantification of protein expression levels at 24 h after HI induction in brain cortex, using GAPDH as control; ** $p < 0.018$; ** $p < 0.09$ vs. sham

Asiatic acid prevented neuronal apoptosis induced by HI in rats

TUNEL staining of brain cortex from HI rats showed a significant increase in neuronal apoptosis, when compared to sham group (Figure 7). However, asiatic acid treatment significantly reduced HI-mediated increase in apoptosis of neurons in rat brain cortex in a dose-based manner, when compared to model group. Indeed, the HI-induced increase in neuronal apoptosis in rat brain cortex was completely suppressed on treatment with asiatic acid at a dose of 20 mg/kg.

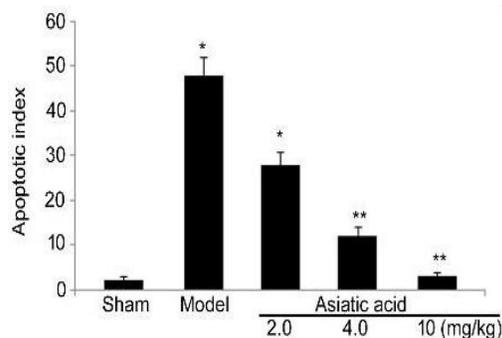


Figure 7: Effect of asiatic acid on neuronal apoptosis in rat brains. The rats were pre-treated with asiatic acid at doses of 2, 4 and 10 mg/kg prior to HI, and neuronal apoptosis in brain cortex was determined using TUNEL assay. Apoptosis index was determined by counting apoptotic cells randomly in five fields; $*p < 0.018$; $**p < 0.09$ vs. sham

DISCUSSION

The TAK1-JNK pathway plays a crucial role in apoptosis induction associated with activated T cells in pulmonary and thyroid cancers [8, 9]. Following HI induction in developing brains, the level of p-TAK1 is increased, leading to brain injury. Therefore, the down-regulation of TAK1 is considered a potential target for treatment of cancers and many other disorders [10]. The present study found markedly higher expression of p-TAK1 in the rat brain cortex at 24 h after HI induction. However, HI-mediated enhancement of TAK1 activation was effectively suppressed in rat brain cortex by pre-treatment with asiatic acid. The HI induction caused a significant increase in proportion of astrocytes containing p-TAK1, and enhanced the population of NeuN containing p-TAK1 in the rat brain cortex. However, asiatic acid treatment alleviated HI, leading to enhancement of pTAK1-positive astrocytes and NeuN-positive cell count in the neonatal rat brain cortex.

Studies on animal models have demonstrated that neuronal apoptosis is crucial in brain injury induced by HI [14]. It has been reported that translocation of apoptosis-activating factors into the nucleus is related to neuronal injury in neonatal rats after hypoxia-ischemia [15]. Brain injury is inhibited by deletion of poly(ADP-ribose) polymerase-1 gene via suppression of nuclear translocation of apoptosis activation factor [16]. Apoptosis inhibition produced significant protective effect against HI-mediated brain injury in neonatal rats [17,18]. In the present study, HI induction markedly promoted neuronal apoptosis in the brain cortex of neonatal rats. Results from TUNEL staining demonstrated that the HI-induced neuronal apoptosis in neonatal rat brain

cortex was effectively alleviated by treatment with asiatic acid. Elevated p-JNK expression was observed in the neonatal rat brain cortex following HI-induction [19,20]. However, asiatic acid markedly reversed the HI-induced up-regulation of p-JNK expression in neonatal rats. The levels of caspase-3, p53 and p-c-Jun were enhanced in the brain cortex of rats following HI. Treatment with asiatic acid significantly suppressed HI-mediated up-regulation of the expressions of caspase-3, p53 and p-c-Jun in rat brain cortex. These findings indicate that asiatic acid alleviated HI-mediated brain injury in neonatal rats via down-regulation of p-TAK1 expression. The asiatic acid-mediated prevention of HI-induced neuronal apoptosis in neonatal rat brain was confirmed using NG25, a TAK1 inhibitor.

CONCLUSION

The findings of this study demonstrate that asiatic acid prevents HI-induced brain injury in neonatal rats via inhibition of neuronal apoptosis. Moreover, asiatic acid inhibits TAK1 activation, suppresses p-JNK expression, and targets pro-apoptotic factors in brain cortex. Therefore, asiatic acid has potentials for use in the management of HI-induced brain injury.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Juan Zhang conceived and designed the study; Ying Wang, Huiping Wang, Pu Zhao, Jiwen Cheng, Wei Gong collected and analyzed the data; Ying Wang, Huiping Wang, while Pu Zhao wrote the manuscript. All authors read and approved the manuscript for publication.

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