

Original Article

Curcumin targets vascular endothelial growth factor via activating the PI3K/Akt signaling pathway and improves brain hypoxic-ischemic injury in neonatal rats

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ABSTRACT This study aimed to evaluate the effect of curcumin on brain hypoxic-ischemic (HI) damage in neonatal rats and whether the phosphoinositide 3-kinase (PI3K)/Akt/vascular endothelial growth factor (VEGF) signaling pathway is involved. Brain HI damage models were established in neonatal rats, which received the following treatments: curcumin by intraperitoneal injection before injury, insulin-like growth factor 1 (IGF-1) by subcutaneous injection after injury, and VEGF by intracerebroventricular injection after injury. This was followed by neurological evaluation, hemodynamic measurements, histopathological assessment, TUNEL assay, flow cytometry, and western blotting to assess the expression of p-PI3K, PI3K, p-Akt, Akt, and VEGF. Compared with rats that underwent sham operation, rats with brain HI damage showed remarkably increased neurological deficits, reduced right blood flow volume, elevated blood viscosity and haematocrit, and aggravated cell damage and apoptosis; these injuries were significantly improved by curcumin pretreatment. Meanwhile, brain HI damage induced the overexpression of p-PI3K, p-Akt, and VEGF, while curcumin pretreatment inhibited the expression of these proteins. In addition, IGF-1 treatment rescued the curcumin-induced down-regulated expression of p-PI3K, p-Akt, and VEGF, and VEGF overexpression counteracted the inhibitory effect of curcumin on brain HI damage. Overall, pretreatment with curcumin protected against brain HI damage by targeting VEGF via the PI3K/Akt signaling pathway in neonatal rats.

INTRODUCTION

Neonatal hypoxic-ischemic encephalopathy (HIE) is a common brain injury disease in near-term infants, with high rates of morbidity, disability, and mortality [1]. Intrauterine hypoxia and intrapartum asphyxia are considered the major underlying causes of HIE and lead to tremendous detrimental effects on brain development in newborns, thereby resulting in devastating disabilities such as mental retardation, cerebral palsy, learning disabilities,

and epilepsy [2-4]. Despite the increased understanding of neonatal disease pathogenesis and the intense advancements in modern treatments, the significant disability and mortality caused by HIE remain serious issues [5,6]. Therefore, elucidating the underlying mechanisms of neonatal HIE to search for effective therapeutic targets is essential.

Various diseases after HIE are mainly caused by long-lasting neurological deficits, and neuroprotective agents are considered potentially therapeutic for neonatal HIE [7]. It has been widely



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recognized that improving blood supply to brain tissue is closely related to the recovery of neuronal function [8]. Vascular endothelial growth factor (VEGF) is a cytokine secreted by vascular endothelial cells and is currently the most important growth factor for inducing angiogenesis [9]. It exhibits neuroprotective and neurotrophic functions *via* promoting proliferation and angiogenesis of vascular endothelial cells, vasodilation, and the synthesis of various vasoactive substances, as well as inhibiting vascular smooth muscle cell proliferation [9]. The application of exogenous VEGF contributes to neuronal survival in mesencephalic explant cultures [10], and VEGF is an attractive target for brain hypoxic-ischemic (HI) damage [11]. Thus, drugs targeting VEGF may be an effective neuroprotective agent for brain HI damage.

In recent years, several traditional Chinese medicines have been demonstrated to exert neuroprotective effects [12,13]. Curcumin, a low-molecular-weight bioactive compound isolated from turmeric (*Curcuma longa*), can affect various diseases by regulating oxidative stress, cell apoptosis, and the inflammatory response [14]. A recent study has suggested that curcumin can attenuate brain HI damage in neonatal rats [15]. However, whether curcumin improves brain HI damage *via* VEGF regulation is still unclear.

The phosphoinositide 3-kinase (PI3K)/Akt signal transduction pathway may be an important signaling pathway for directly regulating VEGF expression in brain microvascular endothelial cells [16]. This study aimed to evaluate the effect of curcumin on brain HI damage in neonatal rats and whether the effect of curcumin is associated with the PI3K/Akt/VEGF signaling pathway.

METHODS

Animal model

This study was approved by the Animal Ethics Committee of Affiliated Hospital of Hebei University prior to experimentation (no. 06ChiC 6 244016475). Healthy neonatal Sprague-Dawley rats (18–25 g, 10 days old, male) were used for the following experiments after one week of acclimation (17-days old). Brain HI damage rat models were established as previously described [17]. Briefly, the rats were placed in the induction chamber and adjust the ether to 4% to induction anesthesia and adjust the ether to 3% to maintenance, then followed by fixation on the operating table. A 0.4 cm cervical incision was cut to expose the arteries. Then the right common carotid artery was ligated with a 7-0 suture, and the skin incision was sutured. After 1 h, rats were placed in an anoxic chamber containing 8% oxygen and 92% ammonia gas for 2.5 h (flow rate 2 L/min), and then returned to the normal oxygen cage. The sham operation consisted of the entire procedure but without ligation and anoxic treatment. Constant temperature was maintained throughout the operation.

Animal grouping, drug administration, and sample collection

In total, 60 neonatal rats were randomly and equally assigned to five groups: 1) the sham group: rats underwent a sham operation and received intraperitoneal injection of saline; 2) the HI group: rats with brain HI damage received intraperitoneal injection of saline; 3) the HI + CUR group: rats received intraperitoneal injection of 200 mg/kg curcumin 30 min before brain HI damage surgery; 4) the HI + CUR + insulin-like growth factor-1 (IGF-1) group: rats received intraperitoneal injection [18] of 200 mg/kg curcumin 30 min before brain HI damage surgery and subcutaneous injection of 3 mg/kg IGF-1 24 h after HI injury (Peptotech, Inc., Rocky Hill, NJ, USA) [19]; 5) the HI + CUR + ov-VEGF group: rats received intraperitoneal injection of 200 mg/kg curcumin 30 min before brain HI damage surgery and intracerebroventricular injection of 20 ng VEGF (Sigma, St. Louis, MO, USA) 5 min after reoxygenation [20]. Hemodynamic measurements were performed 12 h after treatment and the blood pressure during and after HI was monitored to exclude brain injury caused by systemic hypotension. One day (24 h) after treatment, a neurological evaluation was performed and then the rats were sacrificed to obtain brain tissues.

Neurological evaluation

Neurological deficits were evaluated by Longa's method [21] and graded by different scores. Neurological function was graded according to the following scores: 0 (normal, no neurological deficits), 1 (failure to extend left forepaw fully), 2 (turning to the left while walking), 3 (dumping to the left while walking), and 4 (unconsciousness and failing to walk spontaneously).

Hemodynamic measurements

Hemodynamic parameters, including left and right cerebral hemisphere blood flow volume, blood viscosity, and hematocrit, were measured using an electromagnetic blood flowmeter (MFV-3200; Nihon Kohden, Tokyo, Japan) and an auto cleaning rotating viscometer (LBY-N6A; Puli Co., Beijing, China).

Histopathological assessment

Brain tissues of cortex and hippocampus were fixed at 4°C for 24 h with 4% paraformaldehyde, embedded in paraffin, and sliced into sections. After dehydration with gradient ethanol, the sections underwent hematoxylin-eosin (H&E) staining: hematoxylin staining, differentiation in 1% hydrochloric acid alcohol, eosin staining, and mounting with neutral resin. The sections were observed by light microscopy (Olympus, Tokyo, Japan).

2,3,5-Triphenyltetrazolium Chloride (TTC) Staining

The brains were sectioned into spaced coronal blocks (2 mm). These sections were stained with 2% TTC (Coolaber, Beijing, China) at 37°C for 15 min. Images of the staining were acquired using a digital camera (EOS 90D; Canon, Tokyo, Japan). The non-infarcted area was stained red, the infarcted area was grayish white, and the area between the red area and the gray area was ischemic.

TUNEL assay

The HI brain tissue sections of the right brain were obtained as described for HE staining. Then cell apoptosis in brain tissues was evaluated by TUNEL staining using a commercial kit (Roche, Mannheim, Germany) following the manufacturer's guidelines. The sections were observed with a fluorescence microscope (Olympus).

Flow cytometry

Single-cell suspensions of brain tissues in ice-cold flow cytometry buffer (2% fetal calf serum and 2% mouse serum in phosphate buffer saline [PBS]) were prepared. To determine the percentage of early and late apoptotic cells, single cells were stained using the following antibody set: Annexin V-FITC and PI Cells according to the manufacturer's instructions (Cat No: 556547; BD Biosciences, San Diego, CA, USA). Data were acquired on an LSRFortessa cell analyzer (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Western blotting

The HI brain tissue of the right brain in various groups was lysed by RIPA lysis buffer (Gibco, Grand Island, NY, USA) supplemented with phenylmethanesulfonyl fluoride (1 mM, Sigma). Protein was extracted by centrifugation and detected by the BCA kit (Shanghai Sangon Biotech Co., Ltd, Shanghai, China). Then protein samples were separated on SDS-PAGE gel, transferred to polyvinylidene fluoride membranes, and blocked with 5% nonfat milk for 1 h. Next, the membrane was incubated with anti-rat p-PI3K, PI3K, p-Akt, Akt, VEGF, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:500, Cat: sc-32233; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C, washed with PBS, and incubated with secondary antibody (1:5000, Cat:111-035-003; Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at room temperature. Enhanced chemiluminescence (ECL, Millipore, USA) was used to detect the protein levels.

Statistical analysis

GraphPad Prism 6 software (GraphPad Software Inc., San

Diego, CA, USA) was used for statistical analysis. Data were expressed as mean \pm standard deviation and analyzed by one-way ANOVA followed by multiple comparison with the Tukey test. A value of $p < 0.05$ was considered significant and $p < 0.01$ was considered highly significant.

RESULTS

The effect of curcumin on neurological deficits, hemodynamic changes, and histopathological changes in neonatal rats with brain HI damage

All rats with brain HI damage exhibited depressive symptoms, mainly manifested as tonic convulsions and tremors. To investigate whether the pretreatment with curcumin could alleviate the symptoms and pathological changes of brain HI damage, neurological deficits, hemodynamic changes, and histopathological changes in neonatal rats with brain HI damage were measured. TTC staining showed that compared with HI group, the brain HI damage in HI + CUR group is improved (Fig. 1A). Longa's score results found that compared with the sham group, neurological deficit scores were significantly increased in the HI group ($p < 0.01$, Fig. 1B), while pretreatment with curcumin significantly reduced neurological deficit scores in comparison with the HI group at 24 h and 72 h after treatment ($p < 0.01$, Fig. 1B). The hemodynamic results revealed that compared with the sham group, right blood flow volume was significantly reduced and blood viscosity and hematocrit were significantly increased in the HI group ($p < 0.05$, Table 1), while pretreatment with curcumin decreased blood viscosity and hematocrit ($p < 0.05$, Table 1). In addition, HE staining revealed that the cortical and hippocampal cells exhibited large nuclei and little cytoplasm in the sham group, whereas the cortex and hippocampus in the HI and CUR groups both exhibited different degrees of cell swelling, balloon-like changes, sparse cells, widened gaps, disordered cell arrangement, unclear structure, and nuclear pyknosis (Fig. 1C). Compared with the CUR group, cell damage was aggravated in the HI group (Fig. 1C). TUNEL staining and flow cytometry also revealed conspicuously increased apoptotic cells in the HI group compared with the sham group, while pretreatment with curcumin reduced the number of apoptotic cells ($p < 0.01$, Fig. 1D, E). Both the proportions of early and late apoptotic cells in the HI group were significantly higher than those in the sham group; however, pretreatment of curcumin significantly reduced the proportion in the HI + CUR group ($p < 0.01$, Fig. 1E).

The effect of curcumin on the PI3K/Akt signaling pathway and VEGF in neonatal rats with brain HI damage

Considering the effect of curcumin on brain HI damage, the

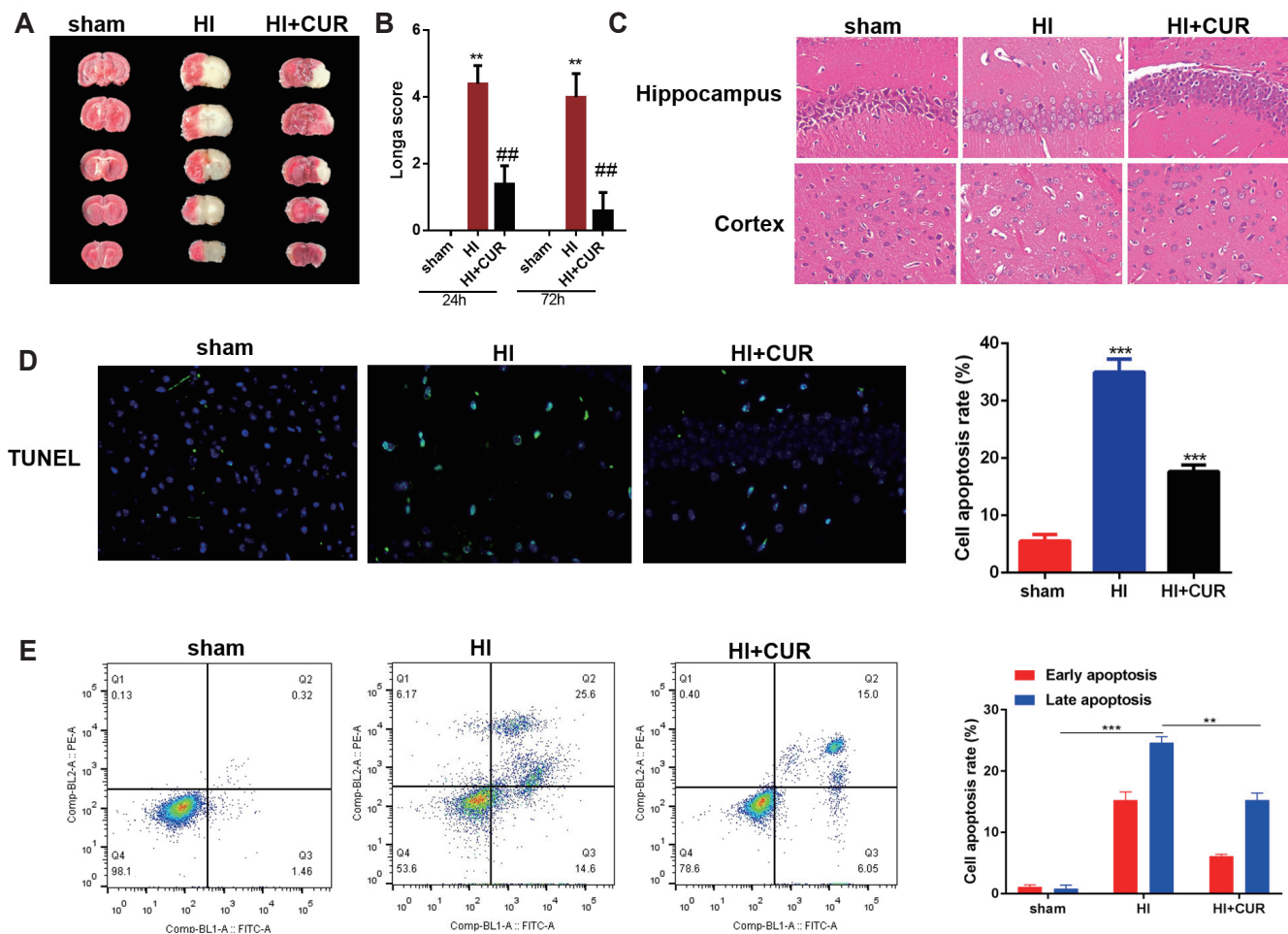


Fig. 1. Curcumin improves brain HI damage in neonatal rats (n = 12 per group). (A) HI damage in brain tissues of the sham, HI, and HI + CUR groups by TUNEL staining. (B) Neurological deficits evaluated in the sham, HI, and HI + CUR groups by Longa's score at 24 h and 72 h after treatment. (C) Histopathological changes of brain tissues in the sham, HI, and HI + CUR groups by H&E staining (×400). (D) Cell apoptosis in brain tissues of the sham, HI, and HI + CUR groups by TUNEL staining (×400). (E) Early and late apoptotic in brain tissues of the sham, HI, and HI + CUR groups by flow cytometry. HI, hypoxic-ischemic injury; CUR, curcumin. **p < 0.01, ***p < 0.001 vs. sham group; [#]p < 0.01 vs. HI group.

Table 1. Haemodynamic changes of rats in the sham, HI, and HI + CUR groups

Parameters	Sham group	HI group	HI + CUR group
Left blood flow volume (ml/min)	46.5 ± 6.3	45.8 ± 5.3	46.2 ± 5.9
Right blood flow volume (ml/min)	47.2 ± 5.6	43.8 ± 4.3*	45.7 ± 5.1
Blood viscosity (mPa·s)	6.05 ± 0.34	7.62 ± 0.29*	6.18 ± 0.24 [#]
Hematocrit (L/L)	0.385 ± 0.002	0.413 ± 0.001*	0.392 ± 0.003* [#]

HI, hypoxic-ischemic injury; CUR, curcumin. *p < 0.05 vs. sham group; [#]p < 0.05 vs. HI group.

expression levels of p-PI3K, p-Akt, and VEGF were analyzed to explore the mechanism of the effect of curcumin. Western blotting revealed that compared with the sham group, the protein expression of p-PI3K, p-Akt, and VEGF were significantly increased in the HI group (p < 0.01, Fig. 2). Pretreatment with curcumin significantly reduced the expression of p-PI3K, p-Akt, and VEGF in comparison with the HI group (p < 0.01, Fig. 2).

The effect of activating the PI3K/Akt signaling pathway in curcumin-treated neonatal rats with brain HI damage

To investigate whether the PI3K/Akt signaling pathway influenced the role of curcumin, curcumin-treated HI rats were administered IGF-1. Compared with the HI + CUR group, IGF-1 treatment significantly promoted p-PI3K, p-Akt, and VEGF expression (p < 0.01, Fig. 3).

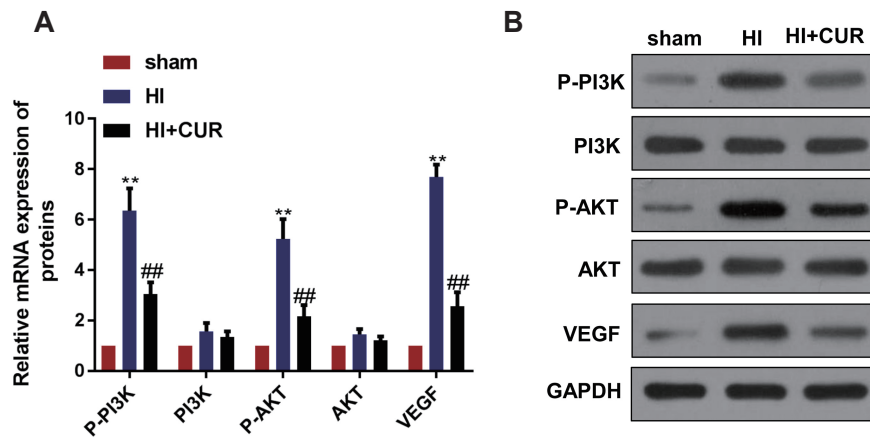


Fig. 2. Curcumin promotes the activation of PI3K/Akt signaling pathway and VEGF expression in neonatal rats with brain HI damage (n = 12 per group). (A) Band intensities from western blots were calculated. (B) The protein expression of p-PI3K, PI3K, p-Akt, Akt, and VEGF in the sham, HI, and HI + CUR groups by western blotting. HI, hypoxic-ischemic injury; CUR, curcumin; VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. ** $p < 0.01$ vs. sham group; ## $p < 0.01$ vs. HI group.

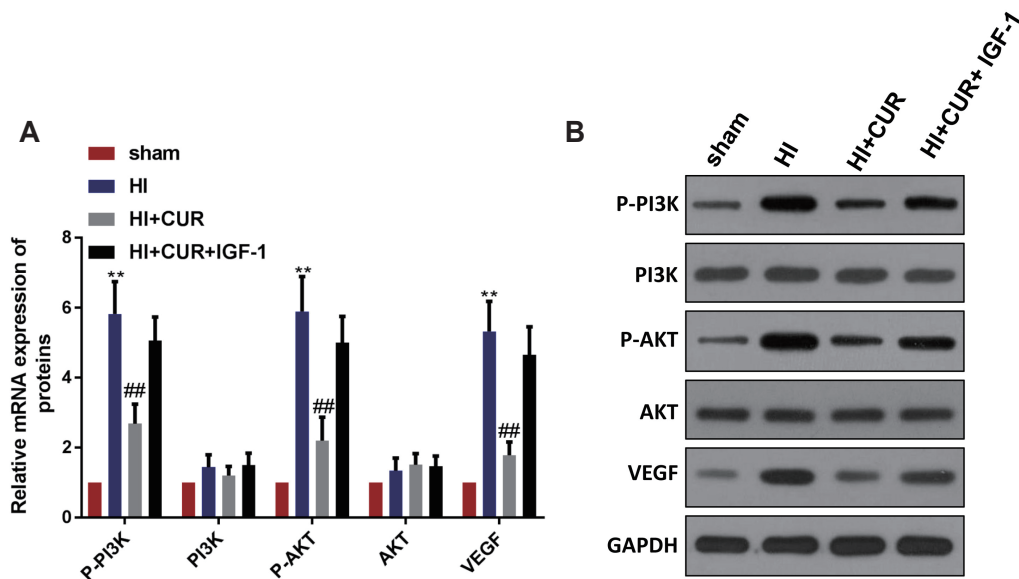


Fig. 3. Inhibitory effect of curcumin on VEGF expression disappears via activating PI3K/Akt signaling pathway in neonatal rats with brain HI damage (n = 12 per group). (A) Band intensities from western blots were calculated. (B) The protein expression of p-PI3K, PI3K, p-Akt, Akt, and VEGF in the sham, HI, HI + CUR and HI + CUR + IGF-1 groups by western blotting. HI, hypoxic-ischemic injury; CUR, curcumin; IGF-1, insulin-like growth factor 1; VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. ** $p < 0.01$ vs. sham group; ## $p < 0.01$ vs. HI group.

The effect of VEGF overexpression in curcumin-treated neonatal rats with brain HI damage

To further explore the role of VEGF in curcumin-treated neonatal rats with brain HI damage, curcumin-treated HI rats were administered VEGF. Also, VEGF expression was higher in the HI + CUR + ov-VEGF group than that in the HI+CUR group (Fig. 4A). The results also showed that compared with the HI + CUR group, VEGF overexpression significantly increased brain HI damage (Fig. 4A), neurological deficit scores at 24 h and 72 h after treatment ($p < 0.01$, Fig. 4C) and also aggravated cell damage (Fig.

4D) and cell apoptosis (Fig. 4E, F).

DISCUSSION

In the present study, a neonatal rat model of brain HI damage was successfully constructed. The results revealed that pretreatment with curcumin significantly improved neurological deficits and hemodynamic changes, attenuated histopathological injury and cell apoptosis, and decreased the number of both early and late apoptotic cells in neonatal rats with brain HI damage.

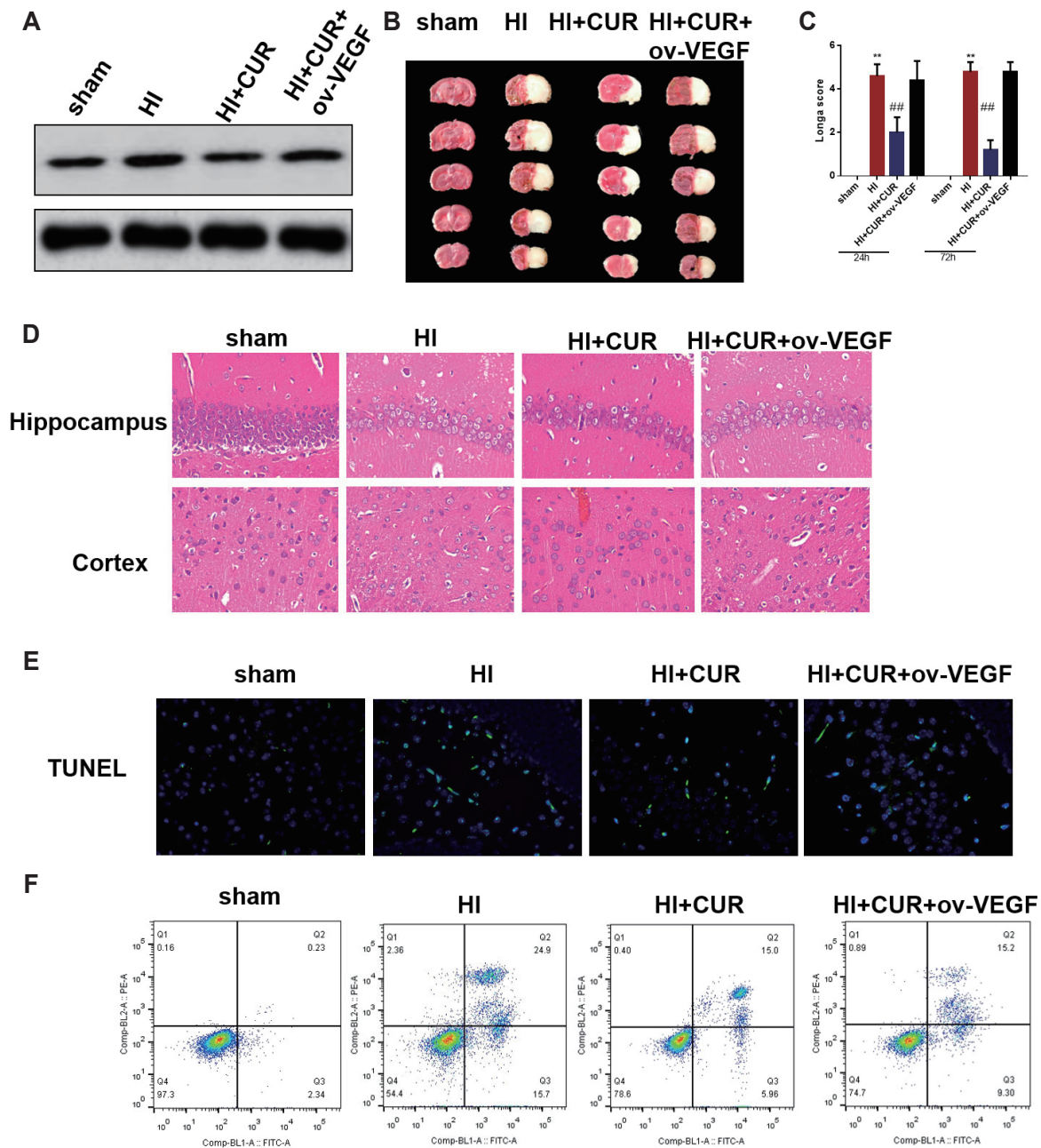


Fig. 4. The inhibitory effect of curcumin on brain HI damage is counteracted by VEGF overexpression in neonatal rats (n = 12 per group). (A) The VEGF expression in the sham, HI, HI + CUR, and HI + CUR + ov-VEGF groups by western blotting. (B) HI damage in brain tissues of the sham, HI, HI + CUR, and HI + CUR + ov-VEGF groups by TUNEL staining. (C) Neurological deficits evaluated in the sham, HI, HI + CUR, and HI + CUR + ov-VEGF groups by Longa's score at 24 h and 72 h after treatment. (D) Histopathological changes of brain tissues in the sham, HI, HI + CUR, and HI + CUR + ov-VEGF groups by H&E staining ($\times 400$). (E) Cell apoptosis in the sham, HI, HI + CUR, and HI + CUR + ov-VEGF groups by TUNEL staining ($\times 400$). (F) Early and late apoptotic in brain tissues of the sham, HI, HI + CUR, and HI + CUR + ov-VEGF groups by flow cytometry. HI, hypoxic-ischemic injury; CUR, curcumin; VEGF, vascular endothelial growth factor. ** $p < 0.01$ vs. sham group; ### $p < 0.01$ vs. HI group.

Moreover, pretreatment with curcumin remarkably reduced the expression of p-PI3K, p-Akt, and VEGF, while IGF-1 rescued the curcumin-induced down-regulated expression of p-PI3K, p-Akt, and VEGF. In addition, VEGF injection counteracted the inhibitory effect of curcumin on brain HI damage.

Curcumin has been demonstrated to improve several neurological diseases, such as subarachnoid hemorrhage, ischemic stroke,

and Alzheimer's disease, via alleviating neuroinflammation and neurological injury [22]. This study found that pretreatment with curcumin alleviated neurological deficits, hemodynamic changes, and histopathological alterations induced by brain HI damage. This suggests that curcumin was able to protect against brain HI damage in neonatal rats. Yu *et al.* [23] have demonstrated that curcumin attenuated hypoxia-hypercapnia brain damage-

induced apoptosis and brain edema in rat models. Wang *et al.* [24] have reported that curcumin attenuates brain injuries induced by chronic intermittent hypoxia via regulating the expression of aquaporin 4 and the p38 MAPK pathway. In addition, Joseph *et al.* [25] have proved that nonencapsulated curcumin exerts effective neuroprotection in neonatal rats with HIE. Our study is consistent with all these previous studies that demonstrate a neuroprotective effect of curcumin on brain HI damage.

Currently, the underlying mechanisms of the action of curcumin during brain HI damage are yet to be elucidated thoroughly. Liu *et al.* [26] have shown that curcumin contributes to mitochondrial biogenesis, which may lead to the preventive effect of curcumin on cerebral ischemia reperfusion injury. Furthermore, blocking the TLR4/MyD88/NF- κ B signaling pathway may be responsible for inhibiting experimental traumatic brain injury by curcumin via attenuating acute inflammatory injury [27]. Thus, the precise mechanisms of curcumin action in brain injury may be different in different models. This study revealed that VEGF injection counteracted the beneficial effect of curcumin on brain HI damage. After brain injury, VEGF expression has been observed in the central nervous system [28,29]. VEGF can also be induced by hypoxia that results in cerebral edema [30]. Several studies have demonstrated that exogenously administered VEGF has neuroprotective effects on brain HI damage in neonatal rats [20] and that VEGF can alleviate neurological damage by inhibiting neuronal and glial damage [31], increasing the subventricular zone size [32], and promoting neurogenesis [33]. Of note, the relationship between curcumin and VEGF has been investigated in several diseases. For example, Pan *et al.* [34] have reported that curcumin reduces cell growth via inhibiting VEGF expression in hepatocellular carcinoma. Lu *et al.* [35] have shown that curcumin can induce human pterygium fibroblast apoptosis via targeting VEGF expression. In addition, another study has also demonstrated that the anti-tumor effect of curcumin is closely associated with the significantly down-regulated VEGF in thoracic aortic aneurysms [36]. All these studies suggest an inhibitory effect of curcumin on VEGF expression, which is consistent with our study. These results indicate that pretreatment with curcumin might attenuate brain HI damage via targeting VEGF.

Furthermore, the present study found that pretreatment with curcumin inhibited the PI3K/Akt signaling pathway, while IGF-1 treatment activated this pathway and VEGF expression. It has been demonstrated before that IGF-1 can activate the PI3K/Akt signaling pathway [37], play an important role in neuronal proliferation and differentiation [38], and exert neuroprotective effects on brain HI damage in neonatal rats [39]. It has also been reported that the PI3K/Akt signaling pathway participates in angiogenesis [39], and that its activation can induce VEGF expression [40]. Our study revealed that activating the PI3K/Akt signaling pathway inhibited the neuroprotective effect of curcumin in neonatal rats with brain HI damage. Similarly, recent studies have also demonstrated that activation of the PI3K/Akt signaling

pathway contributes to the recovery of neurological function after brain HI damage in neonatal rats [41] and neonatal rabbits [42]. Collectively, we postulate that pretreatment with curcumin protects against brain HI damage by targeting VEGF via the PI3K/Akt signaling pathway.

In conclusion, the findings of this study suggest that pretreatment with curcumin improved brain HI injury in neonatal rats, which is likely associated with VEGF via the PI3K/Akt signaling pathway.

However, one limitation of this study is that the more information on the role of VEGF in the effect of curcumin on brain HI damage needs to be explored in the further study. Another limitation of this study is the controversy about the use of curcumin in pregnant women. Hence, further studies will aim to determine the safe dose of curcumin and its optimal administration, especially during pregnancy. In the future, we will also study whether administration of curcumin may rescue individuals in the early stages of hypoxia-induced changes.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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