Neuroprotective effect and mechanism of butylphthalide after cerebral ischemia-reperfusion injury in rats

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Abstract

Introduction: To investigate the neuroprotective effect and mechanism of DL-3-n-butylphthalide (NBP) on the brain-derived neurotrophic factor (BDNF)/tyrosine kinase B (TrkB) and its downstream signalling pathway after cerebral ischemia/reperfusion injury (CIRI) in rats.

Material and methods: The middle cerebral artery occlusion/reperfusion (MCAO/R) model was used. Reperfusion was performed 2 h after ischemia, and 20 mg/kg of NBP was intraperitoneally injected. Neurological defect score and pathological changes were performed. Apoptotic cells were detected using in situ end-labelling with TUNEL. The expression of BDNF and TrkB proteins was measured by Western blot and immunohistochemical staining. BDNF mRNA, TrkB mRNA, protein kinase B (AKT) mRNA and caspase-3 mRNA expression were measured using real-time polymerase chain reaction (qPCR).

Results: After 24 h of reperfusion, the neurological defect score and the percentage of apoptotic cells in the ischemia/reperfusion group (I/R group) were higher than those in the ischemia/reperfusion + drug group (I/R + d group). The positive expressions of BDNF and TrkB mRNA and protein in the I/R + d group were obviously higher than those in the I/R group (p < 0.05). After intervention with the TrkB receptor inhibitor (K252a), the expression levels of BDNF and TrkB and AKT mRNA were significantly decreased in the ischemia/reperfusion + drug + TrkB receptor inhibitor group (I/R + d + R group) compared with the I/R + d group, however the caspase-3 mRNA expression level showed the reverse trend. The expressions of BDNF, TrkB and p-Akt proteins in the I/R + d group were remarkably higher than those in the I/R group at each time point, and reached the peak at 24 hours after reperfusion, which were earlier than that in the I/R group.

Conclusions: Butylphthalide represents a neuroprotective effect after CIRI in rats and used within 24 h of early onset contributes to better prognosis. The underlying mechanism may be related to reducing the apoptosis of nerve cells through BDNF/TrkB signalling pathway.

Key words: butylphthalide, brain-derived neurotrophic factor (BDNF), tyrosine kinase B (TrkB), cerebral ischemia-reperfusion.

Introduction

Stroke is one of the most common diseases in developing countries and one of the leading causes of morbidity and mortality worldwide. Almost 70% of the survivors from stroke have different degrees of disability [11]. In China, with the aggravation of population aging, the risk of cerebrovascular disease is becoming more critical and the incidence of cerebral infarction...
increases the highest one among the prevalence of numerous diseases [20]. Blood flow in the occluded vessels can be restored via intravascular thrombolysis or arterial interventional therapy and neuronal ischemia-reperfusion. Reperfusion after ischemia can salvage the dying cells in the ischemic penumbra. However, various reperfusion therapies can aggravate or even damage the nerve cells, resulting in ischemia-reperfusion injury (IRI) [21]. Currently, neuroprotective drugs for cerebral ischemia-reperfusion injury (CIRI) have become a research hotspot.

DL-3-n-butylphthalide (NBP) is one of the chemical constituents in the oil of celery (Apium graveolens Linn), which is extracted from celery seeds and primarily responsible for the aroma and taste of celery [26]. Studies have shown that NBP can induce the downregulation of intercellular adhesion molecule 1 (ICAM-1) and protease-activated receptor 1 (PAR-1) in cerebrovascular endothelial cells after cerebral ischemia and reperfusion, thereby inhibiting the inflammatory reaction and thrombosis. NBP has been shown to promote the infiltration of neutrophils and inflammatory factors in ischemic lesions, reduce the range of cerebral infarction and improve the function and metabolism of cells [1]. In a previous study, a rat brain injury model was induced after bilateral common carotid artery ligation. NBP effectively alleviated local inflammatory injury and inhibited cell apoptosis through increasing the energy supply of mitochondria and damaged the normal morphology and basic function of mitochondria [22]. NBP can promote the production of corticospinal tract fibres and improve the recovery of neurological function in rats with focal cerebral ischemia [16].

The mechanism of NBP on CIRI is yet to be elucidated. In this study, the neuroprotective effect of NBP was evaluated and its specific mechanism was studied using a cerebral ischemia-reperfusion model.

Material and methods

Materials

All procedures performed on experimental animals were approved by the Animal Care and Use Committee of Sun Yat-sen University and were conducted in accordance with the Guide to the Care and Use of Experimental Animals by the National Research Council (1996, USA).

Animals

One hundred and sixty-two male Sprague-Dawley (SD) rats, weighing 250-300 g, were provided by Sun Yat-sen University Experimental Animal Center (Shenzhen, China).

Main reagent

NBP was provided by Shiyao Group Pharmaceutical Co., Ltd. (Shijiazhuang, China). BDNF, TrkB, p-AKT and caspase-3, terminal deoxynucleotidyl transferase-mediated nick end-labelling (TUNEL) kit, Strept-Avidin-Biotin Complex (SABC) immunohistochemical kit and Western blot reagents were supplied by Boster Biological Technology Co., Ltd. (Wuhan, China). DAB (3,3’-diaminobenzidine) chromogenic reagent kit, wood grain and eosin were supplied by the Department of Pathology, the Seventh Affiliated Hospital. BCA protein quantitative kit, cracking liquid RIPA organization and enhancement fixing kits were purchased from Blue Skies Biotechnology Research Institute (Shanghai, China). Rabbit anti-β-tubulin
antibody, goat anti-rabbit IgG (H + L)-HRP conjugate, 20× LumiGLO Reagent and 20× peroxide were purchased from Cell Signaling Corporation (Shanghai, China). Predye protein Marker (Fermentas, Lithuania), Trizol and real-time polymerase chain reaction (qPCR) kit were purchased from Promega Corporation (Shanghai, China). BDNF, TrkB, β-actin primary antibody and related secondary antibodies were purchased from Sigma Chemical Company (St. Louis, MO, USA). BDNF, TrkB, AKT, caspase-3 and β-actin primers were synthesized by Shanghai Shengon Biological Engineering Co., Ltd. (Shanghai, China).

Methods

Model establishment and screening

One hundred thirty-eight rats were randomly selected from 162 rats for modelling and the other 24 rats were consequently divided into the sham operation group. The protocol used for establishing the middle cerebral artery occlusion/reperfusion (MCAO/R) rat models was adopted from Woodruff et al. [23]. Reperfusion was performed two hours after ischemia. Five of the model rats showed no symptoms of neurological impairment and no obvious manifestation of neuronal ischemia in HE staining, thereby, the model was considered to have failed and the rats were removed. Another seven rats died of asphyxiation after getting stuck in their cages during the escape. The cause of death was not confirmed by pathological examination. In the sham group, only the cervical artery was separated but the fishing line was not inserted. Finally, all the rats were anesthetized by intraperitoneal injection of pentobarbital (45 mg/kg), and brain tissues of the parietal lobe were removed.

In the I/R + d group (60 rats) and ischemia/reperfusion + drug + TrkB receptor inhibitor group (I/R + d + R group) (6 rats), 20 mg/kg-NBP was intraperitoneally injected every day after ischemia-reperfusion until the rats were anesthetized by intraperitoneal injection of pentobarbital (45 mg/kg), and brain tissues of the parietal lobe were removed. Six rats were observed and euthanized under anaesthesia at 6 h, 12 h, 24 h, 48 h, 7 d, and 14 d after reperfusion, respectively (6 rats were randomly selected at each time point). Protein expressions of BDNF, TrkB, p-AKT and caspase-3 were detected with immunohistochemistry.

Neurological function score

Neurological defects were scored using the Zea Longa 5-point scoring technique [30]. Rats in the sham group were scored when awake after surgery and rats in the I/R + d and the I/R groups were scored when awake after ischemia/reperfusion.

The scoring points were as follows: 0 points – no neurological deficits, 1 point – not extending the left forelimb, 2 points – turning to the left when walking, 3 points – dumping to the left when walking, and 4 points – unable to walk spontaneously and losing consciousness. Those with a score of 0 or 4 were excluded and the corresponding numbers for each group were supplemented according to the same random principle.

Preparation and histomorphology observation of the specimen

In the sham, I/R and I/R + d groups, six rats were randomly injected 24 h after reperfusion. The rats were anesthetized deeply by intraperitoneal injection of pentobarbital (45 mg/kg) and then perfused with saline (about 200 ml) and 4% (mass concentration) polyformaldehyde solution (about 400 ml). After soaking in 10% (volume fraction) neutral formalin solution for 24 h, 2 mm brain tissue was cut from the coronal plane before and after chiasma. Paraffin sections were prepared and stained with H&E. Morphological changes of parietal lobe were observed under the light microscope.

Immunohistochemical staining

SABC immunohistochemical method was used to determine the contents of BDNF, TrkB, p-AKT and caspase-3 in the parietal lobe tissue according to the instructions of the kit. The routine paraffin sections were dewaxed with 3% hydrogen peroxide (H₂O₂) in methanol and incubated at room temperature for 10 minutes. After dewaxing with xylene, and rehydration with gradient alcohols, the slides were heated to boiling in 10 mm sodium citrate buffer pH 6.8 for 10 minutes in a microwave oven. After treatment with 3% H₂O₂ in methanol to inactivate the endogenous
peroxidase activity, the samples were blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 20 minutes, and then Rabbit anti-Mouse resistance (1:100) for all night at 4°C. After washing 3-4 times with PBS, the samples were incubated with Sheep anti-rabbit resistance (1:100) at room temperature for 30 minutes, and then rinsed with PBS after each step. Slides were then developed using chromogenic detection, counterstained with haematoxylin and rinsed with 30 mm ammonium hydroxide. The slides were mounted with aqueous or permanent mounting medium. The Aperio Scan-scope XT Slide Scanner (Aperio Technologies, Vista, CA, USA) was used to calculate the mean grey value. The higher the grey value, the weaker the positive expression.

**In situ end-labelling with TUNEL**

Apoptosis was detected using the TUNEL in the first three slices of each group. The specific operation was performed according to the instructions. Each slice was observed under a microscope at 250× magnification. About five visual fields with non-repeated 250× magnifications were selected. The percentage of total apoptotic cells in each field was calculated and the average value was calculated [32].

**Western blot assay**

In the sham, I/R and I/R + d groups, six rats were randomly sacrificed at the end of 24 h of reperfusion. The parietal lobe of the brain tissue was placed into the tissue lysate. The rats were thoroughly homogenized, placed in ice for 30 minutes, then centrifuged at 14000 rpm and 4°C for 30 minutes. The protein was quantified according to the BCA protein assay kit. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 10 μl of the samples, and transferred to a PVDF membrane. The membrane was blocked with 5% skim milk in Tris buffer saline (TBS) Tween-20 for 1 h and then incubated 2 h at 4°C with the primary antibody. After being washed with TBST, the blots were incubated with secondary antibodies (1:5000) for 2 h at room temperature, whereas 20× LumiGLO Reagent and 20× Peroxide reagent kits were used for protein detection. The signals were quantified using quantity one (BiorRad), and the values were normalized to x-tubulin signal and presented as the mean ±SEM.

**Quantitative real-time PCR**

The mRNA expression was measured by real-time polymerase chain reaction (qPCR). Total RNA from ischemic and normal tissues of rats was extracted using TRIzol reagent according to the manufacturer’s instructions. RNA was reverse transcribed into cDNA using a reverse transcription system (Promega, Madison, WI, USA). qPCR was performed on an ABI 7900 PCR detection system using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Parallel amplification of the beta-actin gene was used to normalize the gene expression. The relative expression level of target mRNA was calculated using the ΔΔCt method. PCR primer sequences used in the study are listed in Table I.

**Table I. Primers used in PCR analysis**

| Gene name | Sequence |
|-----------|----------|
| BDNF      | 5'-GTGACAGTATTAGGCCAGTGG-3' |
|           | 5'-TATCTTTATGAACCGCAGCC3' |
| TrkB      | 5'-TGCGCTTCAGTGGTTCTAAC-3' |
|           | 5'-CCGTGAGGGGATTCTATTG-3' |
| AKT       | 5'-GGACTACTTGCACTCCGAGAAG-3' |
|           | 5'-CATAGTGCCACCGTCTGGAC-3' |
| Caspase-3 | 5'-GGAGTCTGACTGGAAAGCCGAA-3' |
|           | 5'-CTTCTGGCAAGCCATCCTG-3' |
| β-actin   | 5'-AGCATTTGGCGTGGACAGATGAGG-3' |
|           | 5'-ATGCCATCCTGGACTGACCTG-3' |

**Statistical analysis**

The data were analysed using SPSS software (version 19.0). The data were expressed by mean ± standard deviation (SD). The Shapiro-Wilk test was used for normality assessment. Levene’s test was used to verify the homogeneity of variance. The mRNA content, protein expression grey value, light density ratio and apoptotic cell number in the rat brain followed the normal distribution and homogeneity variance. However, neurological deficit scores did not follow the normal distribution and homogeneity of variance. A comparison between groups was performed with a one-way variance of analysis (ANOVA). Multiple comparisons of the two sample mean were conducted using the least significant difference (LSD) in ANOVA. The Mann-Whitney U test was used to compare the
neurological deficit score. A value of $p < 0.05$ was considered statistically significant.

Results

Model establishment

There were no neurological deficit symptoms in the sham group. 126 rats in the model group showed different degrees of neurological deficit symptoms, mainly manifested as a mental disorder, unilateral Horner syndrome, left lateral rotation and dumping. The H&E staining in the sham group showed no neuronal ischemia. The cell structure was complete, the cytoplasm and nucleus were clear, the cortical neurons of the model rats were incomplete in structure and the edges were fuzzy. The cytoplasm was shrunken, vacuoles appeared on the edges, nuclei were deeply stained and contracted and nuclear disappeared (Fig. 1). The above evidence indicates the success of the modelling.

Indexes of each group were measured at 24 h of reperfusion

Neurological function score

The neurological function score in the sham group was 0 points. In the I/R and I/R + d groups, there were neurological deficit scores with different degrees. The scores were higher in the I/R group ($z = 15.79$) than in the I/R + d group ($z = 9.21$) and the difference was statistically significant ($p = 0.014$).

Histomorphology and TUNEL detection of neuronal apoptosis

The H&E staining of the brain tissue in the sham group showed no neuronal ischemia. The cell structure was intact and the cytoplasm and nucleus were clear. In the I/R group, there were numerous neurons in the cortex of the rats. The cortex was characterized by pyknosis of the neurons, irregular and deep cytoplasm, small nucleolus or unclear appearance and vacuoles on the edges. The structure of the nerve cells in the I/R + d group was relatively intact and a small number of cells showed hyperchromatism and nucleolysis (Fig. 1).

Apoptotic cells were occasionally seen in the sham group. However, they were increased in the I/R group compared with the sham group ($p < 0.05$). In addition, apoptotic cells were significantly lower in the I/R + d group than in the I/R group ($p < 0.05$) (Fig. 2).

The expressions of BDNF and TrkB in rat brain were detected by Western blot after 24 h of cerebral ischemia and reperfusion

The Western blot results showed that compared with the sham group, the I/R group had an increased expression of BDNF and TrkB in the brain tissue after 24 h of cerebral ischemia-reperfusion, and the difference was statistically significant. Compared with the I/R group, the I/R + d group had an increased expression of BDNF and TrkB protein, and the difference was statistically significant ($p < 0.05$) (Fig. 3).

Butylphthalide inhibits neuronal apoptosis through the BDNF-TrkB-pAkt-Caspase-3 pathway

The qPCR results showed that the expression of BDNF, TrkB and p-AKT mRNA in the brain tissue was increased at 24 h after cerebral ischemia and reperfusion. The difference was statistically significant. Compared with the I/R group, the I/R + d group had an increased expression of BDNF, TrkB and p-AKT mRNA, but reduced expression of caspase-3 mRNA. Moreover, the expression of BDNF, TrkB and p-AKT mRNA decreased and caspase-3 mRNA...
expression increased in the I/R + d + R group compared with the I/R + d group, and the difference was statistically significant (Fig. 4).

Expression of BDNF, TrkB, p-AKT and caspase-3 protein at different time points after cerebral ischemia-reperfusion

The expression of BDNF, TrkB, p-Akt and caspase-3 proteins after cerebral ischemia and reperfusion differed at different time points in the I/R and the I/R + d groups. The differences were statistically significant (Fig. 5). The expression trend of BDNF, TrkB and p-Akt was relatively consistent with the extension of time. BDNF, TrkB, p-Akt and caspase-3 proteins were gradually increased in the I/R group after cerebral ischemia-reperfusion. BDNF, TrkB, p-Akt expression peaked at 48 h after reperfusion and caspase-3 protein expression peaked at 7 d, then decreased gradually. The expression of BDNF and TrkB proteins in the I/R + d group peaked at 24 h after reperfusion, and gradually decreased after reperfusion. The expression of caspase-3 in the I/R + d group decreased gradually after cerebral ischemia-reperfusion and the decrease was marked 24 h after reperfusion. The protein expression of BDNF, TrkB and p-Akt was higher in the I/R + d group than that in the I/R group at different time points; the difference was statistically significant (p < 0.05). The expression of the caspase-3 protein was lower in the I/R + d group than that in the I/R group at different time points, and the difference was statistically significant (p < 0.05) (Figs. 5, 6).

Discussion

Our current work was first to demonstrate that the underlying mechanism of the neuroprotective effect of NBP on CIRI is that inhibiting neuronal apoptosis through activating the BDNF-TrkB signal transduction and its downstream signalling pathway. The neuroprotective effect of NBP on CIRI.
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reached its peak at 24 h, which is earlier than within 48 hours from drug instructions of NBP, and lasted for 14 d, providing innovative evidence for the administration period of NBP in the early stage of cerebral infarction. Therefore, early administration in the acute phase represents an attractive strategy for improving the prognosis of neurological impairment.

BDNF is a protein synthesized in the brain [18]. BDNF monomer is a secreted mature polypeptide composed of 119 amino acid residues. The protein isoelectric point is 9.99 and the molecular weight is 13.5 kDa. It is mainly composed of the beta fold and the random N-terminal structure, containing three two-sulfur bonds. BDNF is an alkaline protein distributed in the central nervous system, peripheral nervous system, endocrine system, bone and cartilage tissue and other extensive areas. However, it is mainly expressed in the central nervous system [3]. BDNF plays a crucial role in neuronal survival, differentiation, growth and development. It prevents neuronal damage and death, improves the pathological status of neurons and promotes the biological effects of regeneration and differentiation of injured neurons [18]. Ramos et al. [15] found that BDNF could promote the recovery of neurological function and the regeneration of oligodendrocytes and myelin sheath in rats with an ischemic subcortical injury. After brain injury, the expression of BDNF mRNA and its specific receptor (TrkB) increased and the trend corresponded with BDNF mRNA, thereby expanding the efficacy of BDNF in the neuroprotective role [27]. In transient middle cerebral artery occlusion (tMCAO) rats, it was observed that enhancing BDNF expression could alleviate oxidative stress induced by cerebral ischemia-reperfusion injury.

**Fig. 3.** Butylphthalide can increase the protein expression of BDNF and TrkB against the apoptosis at 24 h after cerebral ischemia reperfusion. **A** The intensity of BDNF protein bands was assessed by optical density (OD) analysis. **B** Data of BDNF protein are given as mean ±SD gained from six independent animals from each experiment group (Western blot, n = 6); **p < 0.05, ****p < 0.05 vs. sham group, ***p < 0.05 vs. I/R group. **C** The intensity of TrkB protein bands was assessed by optical density (OD) analysis. **D** Data of TrkB protein are given as mean ±SD gained from six independent animals from each experiment group (Western blot, n = 6); **p < 0.05, ****p < 0.05 vs. sham group, ***p < 0.05 vs. I/R group.
by ischemia-reperfusion, prevent brain oedema, protect neuronal apoptosis [8]. These studies indicated that the increase of BDNF could alleviate brain injury and promote the recovery of neurological function. In our study, we detected that BDNF and TrkB mRNA and protein expression were increased after CIRI in rats. Moreover, the damage morphology of nerve cells in the I/R + d group with a high BDNF expression level was relatively mild and the cerebral ischemic area was small, indicating that BDNF has a neuroprotective effect, which was consistent with results from previous studies [8,15].

NBP has several functions that includes anti-coagulant thrombosis [14], reduction of tissue oedema [10], inhibition of apoptosis [5] among other functions. It is a new drug in the field of cerebrovascular under the independent intellectual property right of China. Studies have reported that NBP reduces neutrophil infiltration and inflammatory cytokines and shrinks the infarcted area of ischemic brain tissue [1]. Other studies have confirmed that NBP can promote angiogenesis, increase ischemic blood supply and reduce infarct size, which may be related to the release of nitric oxide (NO) in cell endothelium to promote vascular endothelial proliferation [25]. In the focal cerebral ischemia model, NBP can reduce neurological impairment score, improve the morphological integrity of the white matter and promote neurological function recovery [24]. In the I/R model group, NBP pretreatment can reduce the apoptosis of nerve cells, improve the blood-brain barrier (BBB) permeability, reduce the expression of reactive oxygen species (ROS) in the ischemic area and enhance the expression of superoxide dismutase (SOD) [28]. However, the role of NBP in reperfusion injury after intravenous thrombolysis or arterial interventional recanalization of acute cerebral infarction remains unclear. The effect of early administration of NBP in patients with reperfusion injury remains unclear. Currently, only a limited number of studies have confirmed the neuroprotective effect of NBP, but the exact underlying mechanism remains unclear. In the present study, NBP significantly reduced the morphological damage of nerve cells, the area of ischemic lesions and the nerve function impairment score, indicating the neuroprotective effect of NBP, which is in agreement with previous findings [9,28].

Studies have suggested that the specific neuroprotective mechanism of NBP is associated with inhibiting the caspase activation, reducing the nerve cell injury and releasing the inflammatory mediators [13]. Zhan et al. [29] revealed that NBP could up-regulate the expression of p-AKT, thus improves the permeability of the BBB, thereby plays a neuroprotective role. Through the brain tissue reperfusion model study, Zhao [31] detected that NBP can activate HSP70 gene expression, which may further increase the expression levels of p-AKT and Bcl-2, reduce the expression of Bax, inhibit mitochondrial hyperpolarization, reduce the content of endogenous cytochrome C and apoptosis-inducing factor, and promote the enzyme inhibition of caspase activity, therefore inhibit cell apoptosis. The above-mentioned studies only preliminarily confirmed that NBP regulates nerve cell injury through a single action target, and the opinion remains controversial. Whether NBP regulating nerve cell injury through a specific signalling pathway or gene target is still unclear. In our study, NBP could increase the expressions of BDNF, TrkB and AKT mRNA, and reduce the caspase-3 mRNA expression inversely. Of note, the trending of the above-mentioned mRNA expressions result from NBP was significantly attenuated with the use of TrkB receptor inhibitor (K252a). So far, these notable findings of our current study may...
firstly reveal the reasonable mechanism of the neuroprotective effect of NBP is related to regulating the BDNF/TrkB and its downstream signalling pathway.

Studies [2] have shown that the expression of BDNF was periodic. However, whether the periodic expression of BDNF affects the expression of downstream factors and the specific periodic expression form of BDNF has not been currently reported. In this research, the expression of BDNF, TrkB, p-AKT and caspase-3 protein were varied at different time points after cerebral ischemia-reperfusion. The expression of BDNF, TrkB, p-AKT and caspase-3 protein increased gradually, and peaked after 48 h of reperfusion. The trend of BDNF, TrkB and p-AKT expression kept consistent. This indicated that the expression of BDNF and its downstream factors TrkB and Akt after cerebral ischemia reperfusion in rats was time sensitive, and the expression peak was generally within 48 hours, however, NBP could shorten the peak time to 24 hours. Therefore, our study reasonably speculated that the earlier administration of NBP within 24 hours after cerebral ischemia, reperfusion injury is sufficiently safe and effective.

However, the drawback of this study is that the knockout of BDNF gene was not carried out to confirm the neuroprotective mechanism of NBP. Thus, the exact molecular mechanism underlying the effect of NBP on neuroprotective effects after CIRI does not remain entirely understood. Further research focused on verifying the definite effect and the exact mechanism of targeted BDNF gene knockout is urgently required.

In conclusion, our work provides strong evidence that NBP plays a neuroprotective role by regulating the expression of the BDNF/TrkB signalling pathway and its downstream signalling molecules. Therefore,
Fig. 6. Proteins expression of BDNF, TrkB, p-Akt and caspase-3 at different time points after cerebral ischemia/reperfusion (immunohistochemistry 250× magnification).
early administration in the acute phase suggests an attractive strategy for improving the prognosis of neurological impairment.

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**Disclosure**

The authors report no conflict of interest.

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