Research Report

Neuroprotection of early and short-time applying berberine in the acute phase of cerebral ischemia: Up-regulated pAkt, pGSK and pCREB, down-regulated NF-κB expression, ameliorated BBB permeability

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ABSTRACT

Background: Berberine (BBR) has gained attention for its vast beneficial biological effects through immunomodulation, anti-inflammatory and anti-apoptosis properties. Inflammatory and apoptosis damage play an important role in cerebral ischemic pathogenesis and may represent a target for treatment. The aim of this study was to explore BBR’s effect in ischemic injury and the role of the Akt/GSK (glycogen synthase kinase) signaling cascade in mediating the anti-apoptosis and anti-inflammatory effects in the rat brain of permanent middle cerebral artery occlusion (pMCAO). Male Sprague–Dawley rats were subjected to pMCAO and randomly assigned into four groups: Sham (sham-operated) group, pMCAO (pMCAO+0.9% saline) group, BBR-L (pMCAO+BBR 10 mg/kg) and BBR-H (pMCAO+BBR 40 mg/kg) group. BBR was administered immediately after pMCAO and the neuroprotection was detected. Phospho-Akt (pAkt), phospho-glycogen synthase kinase 3-β (pGSK3β), phospho-cAMP response element binding protein (pCREB), nuclear factor-kappa B (NF-κB) and claudin-5 in ischemic cerebral cortex were detected by immunohistochemistry, reverse transcription-polymerase chain reaction and western blotting. Compared with pMCAO group, BBR dramatically lessened neurological deficits scores, brain water contents and infarct sizes, upregulated the expression of pAkt, pGSK3β, pCREB and claudin-5, and decreased the nuclear accumulation of NF-κB \((P<0.05)\) in ischemic brain. The results showed that BBR reduced ischemic brain injury after pMCAO, and this effect may be via the increasing the activation of Akt/GSK signaling and claudin-5, and decreasing NF-κB expression.

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Introduction

Stroke is the most frequent cause of permanent disability in adults and the third leading cause of death worldwide (Donnan et al., 2008). Despite considerable advances in the understanding of the pathophysiology of acute cerebral ischemia (Lo et al., 2003), therapeutic options for acute ischemia are still limited. Berberine (BBR), an alkaloid derivative from Berberis vulgaris L, has been used extensively in traditional Chinese medicine to treat diarrhea and diabetes. Previous studies have reported that BBR has many beneficial biological effects, including immunomodulation (Kong et al., 2004; Zha et al., 2010), anti-diabetic metabolic effect (Bhutada et al., 2011), anti-inflammatory effect (Kuo et al., 2004), and anti-tumor activity (Tan et al., 2011). Furthermore, studies have shown that BBR exerts a protective effect on several models of cerebral ischemic injury and myocardium injury induced by ischemia/reperfusion (Wang et al., 2004; Zhou et al., 2008). However, the functional role of BBR in acute phase of cerebral ischemia is not clear so far.

After ischemia, depending on the severity of the insult and the ability of the cell to maintain adenosine triphosphate (ATP) synthesis, there are mainly two forms of neuron death, necrosis and apoptosis (Christophe and Nicolas, 2006). The serine-threonine kinase Akt, which is also known as protein kinase B, plays an important role in the cell death/survival pathway. Akt phosphorylation is neuroprotective against ischemic injury (Mangi et al., 2003). Activated Akt phosphorylates several downstream targets of the PI(3)K pathway, including the constitutively active serine-threonine kinase glycogen synthase kinase 3-β (GSK3β). Akt activation upregulates GSK3β and is neuron-specific (Leroy and Brion, 1999). Akt phosphorylates GSK3β on Ser9 to render it inactive, a proposed mechanism for cell death (Frame and Cohen, 2001).

2. Results

2.1. BBR reduced the brain infarction volume

No infarction was observed in the sham-operated group. In pMCAO group, an extensive lesion was developed in lateral cortex (Fig. 2A). The infarct volume was significantly reduced from 44.17±7.44% in pMCAO group to 34.73±2.63% in the BBR-L group (P<0.05) and 30.93±6.66% in the BBR-H group (P<0.01) (Figs. 2A and C) (n=6 in each group).

2.2. BBR attenuated the neurological deficits

Neurological deficit was examined and scored on a 5-point scale and for statistical analysis the Mann–Whitney U-tests were conducted. The neurological deficit scores were significantly lower in pMCAO group (3.50±0.89) compared with Sham control. Berberine decreased these scores in BBR-L (3.13±0.68, P<0.05) and BBR-H (2.88±0.85) groups (10 mg/kg vs. pMCAO group, P<0.05; 40 mg/kg vs. pMCAO group, P<0.01; Fig. 2B). And there is no significant difference between BBR-L group and BBR-H group (10 mg/kg vs. 40 mg/kg, P>0.05).

2.3. BBR decreased the brain water content

Ipsilateral brain water content of Sham group was 78.59±1.31%. Compared with pMCAO control, BBR-L and BBR-H groups showed an intense decline in the percentage of brain water content (pMCAO group vs. BBR-L group: 84.35±1.69% vs. 82.44±1.83%, P<0.05; pMCAO group vs. BBR-H group: 84.35±1.69% vs. 81.84±1.91%, P<0.05) (Fig. 2D) (n=6 in each group).

2.4. BBR upregulated the expression of pAkt, pGSK3β, pCREB and claudin-5 and downregulated the expression of NF-κB (p65)

The expression of pAkt, pGSK3β, pCREB and claudin-5 was upregulated at protein level after systemic administration of BBR in ischemia brain. The localization of pAkt, pGSK3β, pCREB

![Fig. 1 - The chemical structure of berberine.](image-url)
protein was identified by immunohistochemistry at 24 h after pMCAO. Representative immunohistochemistry photographs for pAkt and NF-κB were exhibited in Fig. 3A. As shown in Figs. 3B and C the expressions of pAkt, pGSK3β and pCREB in BBR-L and BBR-H groups (n=6 in each group) were significantly increased at 24 h after surgery vs. pMCAO (P<0.01). Western blotting analyses (Figs. 4A, B) also showed a significant increase of pAkt, pGSK3β and pCREB in BBR-L and BBR-H groups (n=6 in each group) vs. pMCAO (P<0.01).

The expression of NF-κB (p65) was upregulated after ischemia, and was downregulated significantly at mRNA and protein levels after systemic administration of BBR in ischemia brain. In sham operated animals, few cells stained by NF-κB (p65) were seen in the cortex (Fig. 3A5). 24 h after the injury, an intense staining of NF-κB was observed at both cytoplasm and nucleus in the ischemic cortex (Fig. 3A6). In both BBR-L and BBR-H groups, the number of cells labeled with NF-κB (p65) was declined significantly vs. pMCAO (P<0.01) (Fig. 3A7, A8). Compared with pMCAO group, the nuclear NF-κB was significantly decreased in BBR-L and BBR-H groups (both P<0.01) by immunohistochemistry (Figs. 3A, C). In agreement with the results of western blotting (Figs. 4C, D) and RT-PCR (Figs. 5A, B), the expression of NF-κB was significantly decreased in BBR-L and BBR-H groups vs. pMCAO group (both P<0.01).

2.5. BBR protected the integrity of BBB

The integrity of brain blood barrier (BBB) was assessed by claudin-5 which was primarily present in brain tight junctions of endothelia. Compared with pMCAO group, claudin-5’s expression was increased in both BBR-L and BBR-H groups (P<0.01) by western blotting and RT-PCR (Figs. 6A–D) at 24 h (n=6 in each group).

3. Discussion

The past decades have seen unprecedented advances in our understanding of pathophysiological processes in cerebral
ischemia, due in large part to the focus on MCAO as a well-characterized and classical experimental model (Longa et al., 1989; Sun et al., 2003; Yang et al., 2009). Evidences of increased inflammatory and apoptosis damage are observed in this model (Dirnagl et al., 1999; Liu et al., 2009b; Lo et al., 2003). This model allows us to explore BBR’s effect in ischemic injury and the role of the Akt/GSK pathway in mediating the anti-apoptosis and anti-inflammatory effects.

The main finding of the present study is that systemic administration of BBR in a rat pMCAO model 24 h after stroke promoted neurological functional recovery via up-regulating pAkt, pGSK3β, pCREB and NF-κB (p65). Brain samples obtained from Sham group, pMCAO group, BBR-L group and BBR-H group. (A1) The pAkt (Ser473) expression could be observed dominantly in the Sham group at 24 h in the Sham group. (A2) The pAkt (Ser473) expression evidently decreased in the pMCAO group at 24 h than Sham group. (A3, A4) The pAkt (Ser473) expression increased in the BBR-L and BBR-H treatment groups at 24 h than that of pMCAO group. The number of NF-κB positive cells increased in pMCAO group (A6) than Sham group (A5) at 24 h. (A7, A8) NF-κB expression decreased relatively in the BBR-L and BBR-H treatment groups at 24 h. The expression of pAkt (Ser473), pGSK3β (Ser9), and pCREB was increased in BBR-L group (*P<0.01) and BBR-H group (*P<0.01) vs. pMCAO group, and the expression of NF-κB (p65) was decreased in BBR-L group (*P<0.01) and BBR-H group (*P<0.01) vs. pMCAO group. Data are expressed as mean±SD (n=6). One-way ANOVA followed by the LSD test.

**Fig. 3** – Representative immunohistochemistry photographs of NF-κB and pAkt (Ser473) (A) and positive cell number analysis (B and C) of pGSK3β (Ser9), pAkt (Ser473), pCREB and NF-κB (p65). Brain samples obtained from Sham group, pMCAO group, BBR-L group and BBR-H group. (A1) The pAkt (Ser473) expression could be observed dominantly in the Sham group at 24 h in the Sham group. (A2) The pAkt (Ser473) expression evidently decreased in the pMCAO group at 24 h than Sham group. (A3, A4) The pAkt (Ser473) expression increased in the BBR-L and BBR-H treatment groups at 24 h than that of pMCAO group. The number of NF-κB positive cells increased in pMCAO group (A6) than Sham group (A5) at 24 h. (A7, A8) NF-κB expression decreased relatively in the BBR-L and BBR-H treatment groups at 24 h. The expression of pAkt (Ser473), pGSK3β (Ser9), and pCREB was increased in BBR-L group (*P<0.01) and BBR-H group (*P<0.01) vs. pMCAO group, and the expression of NF-κB (p65) was decreased in BBR-L group (*P<0.01) and BBR-H group (*P<0.01) vs. pMCAO group. Data are expressed as mean±SD (n=6). One-way ANOVA followed by the LSD test.
transcription factors IKK-α and IKK-β. And it can phosphorylate and inactivate GSK3β at its N-terminus (at Ser9), and activating CREB phosphorylation, which regulates the expression of genes critical for survival, such as brain-derived neurotrophic factor. In addition, Akt also has a role in modulating intracellular glucose metabolism, and consequently enhances energy production after ischemia. Thus, Akt is an excellent therapeutic target for preserving neuron viability in the acute ischemic period. There are two mammalian isoforms for serine/threonine kinase GSK3: GSK3α and GSK3β. Unlike most kinases, GSK3 is constitutively active in cells and can be inactivated by phosphorylation (Cohen and Frame, 2001). Under stimulation, GSK3β is phosphorylated at serine 9 for GSK3α or serine 9 for GSK3β, resulting in the inhibition of GSK3 kinase activity. Akt phosphorylates GSK3 at both of these sites. Under conditions when Akt activity is increased, GSK3β can be inactivated (Cohen and Frame, 2001). GSK3β is highly expressed in the central nervous system (Leroy and Brion, 1999). Inhibition of GSK3β has been demonstrated to reduce apoptosis and enhance cell survival (Pap and Cooper, 1998). GSK3 has emerged as a key regulatory switch in the modulation of inflammation (Martinez et al., 2002). The present study showed that BBR’s protective effects may be through activation of Akt/GSK signaling pathway.

Our previous studies have proved that the expression of pCREB is upregulated in the brain cortex at early stage of brain ischemia (Liu et al., 2010). Ischemic spinal cord injury induced the CREB phosphorylation at the anterior horn of the spinal cord. CREB phosphorylation was marked in the peri-infarct area (Irving et al., 2000). CREB is abundant in the brain and particularly in neurons. Various extracellular stimuli cause phosphorylation of CREB by protein kinase A, protein kinase B (PKB), extracellular signal-related protein kinase, and calcium–calmodulin-dependent protein kinase. Recently CREB phosphorylation has been found to be crucial in neurotrophin-mediated neuron survival (Finkbeiner, 2000). In our study, it was interesting that the expression of pCREB was increased after ischemic stroke, and BBR can further intensify its phosphorylation. So it was possible that BBR treatment promotes cell survival and decrease inflammation through Akt/GSK activation pathway and pCREB activation cascade.

Inflammation is proved playing a role in human stroke and its animal models several hours after ischemia (Iadecola and Alexander, 2001). Apoptosis is another main mechanism of neuronal death in stroke models evidenced by the ischemia study using transgenic and knockout mice (Dirmagl et al., 1999; Lo et al., 2003). Transcription factor NF-κB plays a pivotal role
in the regulation of immune and inflammatory responses (Liu et al., 2009a; Tak and Firestein, 2001). Particularly in the brain, NF-κB regulates the expression of both proinflammatory genes, such as IL-1β, COX-2, tumor necrosis factor-α, matrix metalloproteinase-9 (MMP-9), and inducible nitric oxide synthase, and genes related to apoptosis such as Bcl-2, manganese superoxide dismutase (Mattson and Camandola, 2001). For pMCAO it was demonstrated that the role of NF-κB was detrimental (Nurmi et al., 2004). In our study, ischemia induced NF-κB nuclear accumulation was ameliorated by BBR administration.

After the onset of cerebral ischemia the cytotoxic response occurs within minutes and encompasses oxidative stress, inflammatory responses, cell death, and neurologic injury (Ji et al., 2012; Liu et al., 2009b; Yang et al., 2009). And the protein levels of pAkt, pGSK, pCREB and NF-κB nuclear accumulation are implicated in the early stage of ischemia (Liu et al., 2010; Sun et al., 2011; Wang et al., 2010). So that original administration of BBR will be effective in the acute phase of cerebral ischemia. Although the clinical application of berberine is not limited by the short therapeutic window, it is better to be applied in the acute phase. Numerous animal trials have demonstrated that berberine administration both from 7 days prior to ischemia and 1 day later showed a neuroprotective effect (Benaissa et al., 2009; Hong et al., 2012; Zhou et al., 2008). And in our study berberine was administrated immediately after the onset of ischemia. Prior research indicated that the potent stroke-protection effect of BBR occurred via reducing MMP-9 activity (Hong et al., 2012), blockade on K+ currents (Wang et al., 2004), decreasing reactive oxygen species level (Zhou et al., 2008), COX-2 expression and PGE2 production (Yoo et al., 2008). In our study, we investigated BBR’s effect on stroke and the close
relationship between BBR and Akt/GSK signaling. BBR administration, both dosage of 10 mg/kg and 40 mg/kg, could relieve nerve defection, reduce brain edema, and decrease infarct size. BBR also could markedly promote the activation of Akt/GSK signaling, increase CREB phosphorylation and NF-κB translocation from cytoplasm to nucleus. The expression of pCREB in brain ischemic was known as a protective role for brain. And the NF-κB translocation was crucial for inflammation progression. Thus, activating Akt/GSK signal pathway may be an attractive candidate to explain protective effects of BBR in the acute stage of ischemic stroke. As a salt form, BBR is thought to be water-soluble. Thus it could be difficult for BBR to pass the BBB under physiological conditions. However, it is suggested that BBR could have a direct action on neuron and accumulate in the hippocampus and permeable to the brain in many brain diseases (Wang et al., 2005). In rat model of Alzheimer’s disease, BBR can ameliorate the spatial memory impairment and increase the expression of interleukin-1β and inducible nitric oxide synthase (Zhu and Qian, 2006).

Edema is the major cause of cerebrovascular death within the first week after stroke. And disruption of the BBB integrity is an early and prominent event in brain ischemia (Petito, 1979). Tight junctions are well-developed between adjacent endothelial cells in the blood vessels of central nervous system, and play a primary role in establishing the BBB (Mark and Davis, 2002). Claudin-5, a major cell adhesion molecule of tight junctions in cerebral endothelial cells, was important for BBB integrity study (Nitta et al., 2003). In our research, the expression of claudin-5 was promoted, and this may be the major reason for BBR’s brain edema reduction effect.

BBR decreased neurologic impairment and tissue injury after cerebral ischemia and this effect may be through activation of Akt/GSK pathway and up-regulation of claudin-5 expression. These results in particular indicate that Akt/GSK signaling might be an attractive therapeutic target for BBR during cerebral ischemia and ameliorating brain injury in stroke.

4. Conclusions

In conclusion, our study provides beneficial evidences for early administration of BBR and its underling mechanisms after acute brain ischemia. Specifically, the protective effect of BBR against ischemic injury may be through Akt/GSK signaling activation, upregulation of pCREB, downregulation of NF-κB nuclear transposition and ameliorated BBB permeability.

5. Experimental procedures

5.1. Animals and ischemia protocol

All procedures were performed in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. The experimental protocol was approved by the institutional animal care and use committee and the local experimental ethics committee and conformed to internationally accept ethical standards. All efforts were made to alleviate animal suffering, to minimize the number of animals used, and to utilize alternatives to in vivo techniques, if permitted. Male Sprague–Dawley rats (250–280 g) were kept on a 12 h light/12 h dark regime, with free access to food and water, which were supplied by the Laboratory Animal Centre, Hebei Medical University, Shijiazhuang, Hebei, China. Ninety-six rats were randomly divided into four groups (24 rats in each group): Sham operated group that received equal volume 0.9% NaCl (Sham); pMCAO group that received equal volume 0.9% NaCl after pMCAO (pMCAO); low dose group that received BBR at 10 mg/kg after pMCAO (BBR-L); and high dose group that received BBR at 40 mg/kg after pMCAO (BBR-H).

A standard model of pMCAO was used to make permanent focal ischemia as previously described (Longa et al., 1989; Yang et al., 2009). Briefly, rats were anesthetized by 10% chloral hydrate (350 mg/kg, intraperitoneal). In anesthetized rats, the right side of common carotid artery was exposed and isolated. Middle cerebral artery (MCA) was occluded by inserting a monofilament nylon suture with a heat-rounded tip into the internal carotid artery, which was advanced further until it closed the origin of the MCA. Body temperature was monitored and maintained at 36.5 °C to 37.5 °C throughout the surgery. Sham-operated control rats received the same surgical procedure without inserting a filament.

5.2. Drug administration

BBR (Nanjing Zelang Medical Technology Co. Ltd, Nanjing, Jiangsu, China) with purity of more than 98%, was dissolved in saline to prepare concentration of 10 mg/ml. Rats were treated with BBR by intraperitoneal injection at different doses of 10 and 40 mg/kg (added with 0.9% saline to a total volume of 1 ml) per day immediately after cerebral ischemia. In the cases of the pMCAO and Sham group, equal volume 0.9% saline was administered in the same manner.

5.3. Evaluation of neurological deficit

Neurological deficit scores were evaluated by an examiner blinded to the experimental groups at 24 h after pMCAO following a modified scoring system based on that developed from Longa et al. (1989) and Ding et al. (2002), as follows: 0, no deficits; 1, difficulty in fully extending the contralateral forelimb; 2, unable to extend the contralateral forelimb; 3, mild circling to the contralateral side; 4, severe circling; and 5, falling to the contralateral side. The higher the neurological deficit score, the more severe impairment of motor motion injury. The rats were from groups of brain water content, infarct volume, immunohistochemistry, western blot and reverse transcription-polymerase chain reaction (n = 24).

5.4. Measurement of brain water content

Brain water content was measured by the standard wet/dry weight method. Six rats of each group were deeply anesthetized with 10% chloral hydrate and killed by decapitation at 24 h after pMCAO. The brains were quickly removed and placed on a cooled surface. A coronal brain slice (about 3 mm thick) was cut and the slice was divided into the ipsilateral and contralateral hemispheres after dissecting free 4 mm frontal pole. The two hemisphere slices packaged with pre-weighted tin foils were immediately weighed on an electronic balance to obtain the wet
weight, and then weighed again to obtain the dry weight after dried for 24 h in an oven at 100 ºC. Brain water content was calculated with the equation as follows: Brain water content (%) = (wet weight-dry weight)/(wet weight-tin foil weight) × 100%.

5.5. Measurement of infarct volume

Infarct volume after pMCAO was determined by 2,3,5-triphenyltetrazolium chloride (TTC) at 24 h after pMCAO. Also 6 rats of each group were euthanized with chloral hydrate and the brains were collected quickly. Then the brains were sliced into 5 coronal sections 3-mm thick each and stained with 2% solution of TTC at 37 ºC for 20 min, followed by fixation with 4% paraformaldehyde. The normal tissue was stained deep red while the infarct area pale gray. Stained slides were then washed with PBS containing 0.1% Tween-20 (TPBS) (10 min×3) each time and subsequently incubated with fluorescence labeling second antibodies (IRDye® 800-conjugated goat anti-rabbit or anti-mouse IgG, 1:5000 dilution) for 30 min. Slides were then rinsed with TPBS (10 min×3) and then dehydrated in gradient alcohol, embedded in paraffin. Coronal brain slices containing third ventricle and basal ganglia about 4 mm thick were cut after dissecting free 7 mm frontal pole (3 mm of which were used for paraffin-embedded sections. The slices were incubated with rabbit polyclonal antibody anti-Akt (1:800, Cell Signaling Technology), anti-pAkt (1:500), rabbit monoclonal antibody anti-GSK3β (1:1000, Cell Signaling Technology), anti-pGSK3β (1:1500), NF-κB (1:500), claudin-5 (1:200) and anti-β-actin (1:500, Santa Cruz Biotechnology). The second day, membranes were washed with PBS containing 0.1% Tween-20 (TPBS) (10 min×3) each time and subsequently incubated with fluorescent labeling second antibodies (IRDye® 800-conjugated goat anti-rabbit or anti-mouse IgG, 1:5000 dilution, Rockland, Gilbertsville, PA) for 1 h at room temperature. Membranes were then again washed with TPBS (10 min×3) and the relative density of bands was analyzed on an Odyssey infrared scanner (LI-COR Bioscience, USA). The densitometric values were normalized with respect to the values of β-actin immunoreactivity to correct for any loading and transfer differences between samples. Six rats each group were used.

5.6. Immunohistochemistry

Brains (n=6 in each group) at 24 h after pMCAO and deep anesthetization were removed and immersed in 4% paraformaldehyde over 24 h at 4 ºC, and then dehydrated in gradient alcohol, embedded in paraffin. Coronal brain slices containing third ventricle and basal ganglia, were used. Standard histological processing was performed for paraffin-embedded sections. The slices were incubated with 3% H2O2, 3% normal goat serum and incubated with interest primary antibodies respectively in 0.01 mol/L phosphate-buffered saline overnight. Rabbit monoclonal antibody against pGSK3β (1:100, Santa Cruz Biotechnology), mouse monoclonal antibody against pAkt (1:50, Cell Signaling Technology), rabbit polyclonal antibody claudin-5 antibody (1:200, Santa Cruz Biotechnology) and rabbit polyclonal antibody NF-κB p65 (1:100, Santa Cruz Biotechnology) were used to detect the expressions. Immunohistochemistry was performed via the avidin-biotin technique, and then hematoxylin staining was selected as counterstaining. The secondary antibodies, secondary biotinylated conjugates and dianinobezidine were from the SP kit (Zhongshan Biology Technology Company, China). Slides were viewed and photographed with a ×400 light microscope (Nikon, Japan).

5.7. Western blot

The cytosolic and nuclear protein and total protein were extracted respectively from rat ischemic and control cortex following the manufacturer’s protocols (Applygen Technologies Inc., Beijing, China) at 24 h after pMCAO. Protein concentration of the supernatant was determined using a BCA Protein Assay Reagent Kit (Novagen, Madison, WI, USA) with bovine serum albumin as the standard. An equivalent amount of 50 μg total protein samples, as well as 40 μg cytosolic or nuclear samples, was separated respectively by sodium dodecyl sulfate-polyacrylamide gels prior and transferred 2 h on to PVDF membranes (Millipore Corporation, USA). After blocking 1 h with 5% non-fat dry milk in phosphate buffered saline (PBS), membranes were incubated overnight at 4 ºC with rabbit polyclonal antibody anti-Akt (1:800, Cell Signaling Technology), anti-pAkt (1:500), rabbit monoclonal antibody anti-GSK3β (1:1000, Cell Signaling Technology), anti-pGSK3β (1:1500), NF-κB (1:500), claudin-5 (1:200) and anti-β-actin (1:500, Santa Cruz Biotechnology). The Odyssey infrared scanner (LI-COR Bioscience, USA). The densitometric values were normalized with respect to the values of β-actin immunoreactivity to correct for any loading and transfer differences between samples. Six rats each group were used.

5.8. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was used to analyze the levels of claudin-5, NF-κB and GAPDH mRNA. At 24 h after pMCAO, rats were reanesthetized and brains were removed and frozen in liquid nitrogen. Total RNA from ischemia cortex was extracted from the brain (n=6 in each group) after pMCAO using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions, cDNA was synthesized using ThermoScript RT-PCR System (Invitrogen). Forward and reverse primers are as follows: NF-κB: 5′-AGAGAAAGCAGCAAGTACACTAAG-3′ and 5′-CAGGGCTTATA-GAAGCCATC-C-3′; claudin-5: 5′-CAGGAGGGGTCGTGTGTGAT-3′ and 5′-AAGGTTAGGCGCA-GTTTGGT-3′; GAPDH: 5′-ACAGCCAA-CAGGGTGTTGGGAC-3′, and 5′-TTTGGAGGTTGCGAGGAACTT-3′. Reverse transcription was carried out using RevertAid first Strand cDNA Synthesis Kit (Fermentas International Inc, Canada) following the manufacturer’s instructions. The polymerase chain reaction was performed in a total volume of 20 μL using GoTaq®Green Master Mix (Promega, Madison, WI, USA). GAPDH was used as an internal standard gene. The RT-PCR products were separated on 2% agarose gel and the intensity of each band was quantified using gel SmartView analysis software (Cognex, Natick, MA, USA). Results were expressed relative to the corresponding intensity of the GAPDH bands from the same RNA sample.

5.9. Data analysis

Group data in this study was analyzed using SPSS 13.0 software. Quantitative data was represented as mean±SD. Statistical analysis was performed by One-way ANOVA followed by LSD test for intergroup comparisons. For neuro- logical deficits, Mann-Whitney U-test was used for comparison between two groups. Differences with P>0.05 were considered statistically significant.
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