

Neuroprotective Effect of Activated Protein C on Blood–Brain Barrier Injury During Focal Cerebral Ischemia/Reperfusion

Dose-Response:
An International Journal
April–June 2020:1–6
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DOI: 10.1177/1559325820917288
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Jinqiao Wang¹, Gaofeng Rao¹, Yifan Ma² , Jingjing Zhang²,
Jingjing Shen³, and Chaohong Shi¹

Abstract

Although the effect of activated protein C (APC) on neuronal injury and neuroinflammatory responses has been extensively studied, the detailed mechanism underlying APC-protective effect in the blood–brain barrier (BBB) injury during ischemia is still not clear. In this study, the APC effect against neuroinflammatory responses was evaluated in the model of right middle cerebral artery occlusion in male Sprague–Dawley rats with 2 hours of ischemia and 22 hours of reperfusion. The results showed that APC can significantly improve the neurological function scoring and reduce the infarct volume and BBB permeability. Moreover, the expression of protein nuclear factor-kappa B (NF-κB), both in cytoplasm and nuclei, was reduced. The downstream of NF-κB activation, including tumor necrosis factor-α and interleukin-1β secretion, was inhibited. In all, APC exerts a neuroprotective effect in focal cerebral ischemia–reperfusion in rats by inhibiting the activation and nuclear translocation of NF-κB. It may indicate a therapeutic approach for ischemic brain injury.

Keywords

activated protein C, cerebral ischemia, NF-κB, blood–brain barrier

Introduction

Stroke has given rise to a heavy financial burden on the society and families due to its high morbidity, mortality, and disability rates. In recent years, ischemic stroke has been one of the main diseases that threatened human health.^{1–3} For the past 2 decades, tissue-type plasminogen activator is the only drug approved by the US Food and Drug Administration for treating acute ischemic stroke. However, tissue-type plasminogen activator has many limitations, which restrict its potential to be applied widely. Such limitations include its relatively short therapeutic time window, the low recanalization rates in large-vessel occlusion, and the risk of intracerebral hemorrhage. In recent years, the effectiveness of mechanical thrombectomy in the treatment of acute ischemic stroke has been greatly improved; however, the rate of functional independence after thrombectomy is low compared to the recanalization rate.⁴ A neurovascular unit (NVU) is a dynamic structure consisting of cerebral microvasculature, extracellular matrix, astrocyte end-feet, pericytes, neurocytes and their axons and other sustentacular cells (eg, microglia and oligodendrocytes), and so on.^{5,6} Neurovascular unit is the basic unit that maintains the normal function of the central nervous system. Its major

functions are regulating brain nutrient supply and biological signal transduction and protecting the brain by preventing harmful substances in the blood from entering the brain. Inflammatory response is one of the essential mechanisms underlying secondary injuries from focal cerebral ischemia which is involved in the whole process of focal cerebral ischemic injury. Inflammatory response participates in cerebral ischemic injury when the cerebral blood flow is occluded due

¹ Department of Rehabilitation Medicine, The First People's Hospital of Wenling, Wenzhou Medical University, Wenling, China

² Department of Chemical and Biomolecular Engineering, The Ohio State University, Columbus, OH, USA

³ School of Civil Engineering & Architecture, Taizhou University, Taizhou, China

Received 16 January 2020; received revised 15 February 2020; accepted 17 February 2020

Corresponding Author:

Chaohong Shi, Department of Rehabilitation Medicine, The First People's Hospital of Wenling, Wenzhou Medical University, No. 333, Chuan'an South Road, Wenling, Zhejiang 317500, China.

Email: wlchaohs@163.com



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to thrombosis or embolism.^{7,8} As a result, NVUs are damaged, neuronal activity and cerebral blood flow are uncoupled, and nerve cell death occurs.

Activated protein C (APC) is a plasma serine protease with functions of antithrombosis, anti-inflammation, antiapoptosis, and cell signal transduction.⁹⁻¹¹ Researchers found that APC can prevent brain cells from programmed cell death, promote nerve cell regeneration, and relieve neuroinflammatory responses. Currently, studies on its therapeutic effects in clinical ischemic stroke are ongoing.¹² Given that the detailed mechanisms of APC are still not clear, especially its protective functions in the blood-brain barrier (BBB) has not been evaluated, this study aims to study the effects of APC on BBB injury due to inflammatory responses after focal cerebral ischemia-reperfusion at the organismal level.

Materials and Methods

Subjects and Groups

This study was approved by the ethics committee for Research Animals of the Affiliated Wenling Hospital of Wenzhou Medical University. The male Sprague-Dawley rats weighted 300 to 330 g and aged 3 months were randomly divided into 3 groups each consisting of 6 to 8 animals: (1) Sham operation group: Normal saline was given, 1 mL/kg; (2) solvent control: normal saline, 1 mL/kg; (3) APC group: 2 mg/kg (Batch # 090m01549v, P2200; Sigma-Aldrich, St. Louis, MO). The above drugs were intraperitoneal injected, respectively, at 6 hours after model establishment.

Model Establishment Methods and Success Criteria

The right middle cerebral artery occlusion (MCAO) model was established with the suture method, with 2-hour ischemia and 22-hour reperfusion. The model was successfully established if there was a flexion of the left forelimb when the tail was lifted after model preparation. The model was successfully established in all rats in the model groups. No death occurred in the sham operation and APC groups. Two rats had died at 24 hours after model establishment in the solvent control group.

Experimental Methods

Neurological function scoring. At 24 and 72 hours after the start of ischemia, the modified Neurological Severity Score proposed by Strebe et al¹³ was used to score neurological function in the 3 groups of rats. This scoring process included the presence of several specific reflexes and completion of motor and behavioral tasks such as beam walking tests, beam balance tests, and voluntary movement tests. The total points were 18. Scoring was completed by a researcher blinded to the grouping.

2,3,5-Triphenyltetrazolium chloride staining and measurement of infarct volume. After 72 hours of ischemia, the animals were anesthetized by intraperitoneal injection of 10% chloral hydrate (400 mg/kg) and then decapitated. The brain tissue was

collected immediately and was examined to exclude subarachnoid hemorrhage. The brain tissue was cut into 2-mm coronal sections, which were incubated in 37°C water with 1% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, St. Louis, MO) solution for 20 minutes in the dark. Then, these sections were fixed in 10% formalin solution for 10 minutes. Photos were taken for 6 TTC-stained brain slices and saved for the measurement of the injured regions. ImageJ software was adopted to calculate the areas with cerebral ischemic injury and that without TTC staining. The total infarct volume was calculated by multiplying the total unstained areas of the 6 brain slices by the thickness of brain slices.

Assessment of BBB permeability. After 2 hours of ischemia and 70 hours of reperfusion, the rats in these groups were anesthetized with 10% chloral hydrate (400 mg/kg), and some of the rats were injected with 3% Evan's Blue (EB) solution 2 mL/kg through the femoral vein. The hearts of these rats were perfused with normal saline after equilibrating for 2 hours, and ischemic brain tissues were collected and kept. The brain tissues were placed into a glass vial and were weighted. Subsequently, 5-mL formamide was added into the vial, which was incubated at room temperature protected from light for 72 hours. The extract was absorbed at 4°C and was centrifuged at 12 000g for 15 minutes. The supernatant was transferred to a 96-well plate. Fluorescence intensity was measured with a multimode reader with an absorption wavelength of 620 nm and an excitation wavelength of 683 nm. The extracted tissues were placed into an oven at 120°C and dried for 24 hours; then, they were weighted again. The EB contents of tissues were calculated according to EB-standardized curves and expressed as $\mu\text{g/g}$ tissue.

Detection of expression of inflammatory factors via Western blot. At 72 hours after the cerebral ischemia, the animals were anesthetized with 10% chloral hydrate and then decapitated to immediately take out their brain tissues. Coronal sections were collected at 3 mm anterior to the frontal pole. The thickness of brain slices was 4 mm. Then, a longitudinal incision was made at 2 mm to the right of the middle line, and brain tissues were collected from the right hemisphere. After the homogenate of collected brain tissues was made, cytoplasm and nuclear proteins were separated. Proteins in the tissues were detected with bicinchoninic acid kits (P1511; Applygen Technologies Inc, Beijing, China). Forty micrograms of proteins were loaded to polyacrylamide gel electrophoresis with the currents of 15 mA/25 mA (stacking gel/separating gel) for 2 hours; electrophoretically transferred onto a polyvinylidene difluoride membrane at 100 mA over 1.5 hours (Immobilon; Millipore, Burlington, MA) that was blocked with 3% skimmed milk powder for 1 hour. Then, primary antibodies were added (NF- κB p65 antibody, 1:2000, Abcam 16502; I κB antibody, 1:1000, Abcam 32518; tumor necrosis factor [TNF]- α antibody, 1:500, Abcam 6671; interleukin [IL]-1 β antibody, 1:5000, Abcam 9722; β -actin antibody, 1:5000, Abcam 8226; and TBP antibody, 1:1000, Abcam 63766). The membrane was washed for 3 times after overnight incubation at 4°C.

Table 1. Activated Protein C Improved Nerve Function Scoring After Focal Cerebral Ischemia–Reperfusion in Rats.

Group	Nerve Function Scoring (Points)	
	24 hours	72 hours
Sham operation group (n = 8)	1.0 (0.6–1.4)	0.8 (0.4–1.1)
Solvent control group (n = 8)	7.9 (6.7–9.1) ^a	7.3 (6.4–8.1) ^a
APC group (n = 8)	6.4 (5.6–7.1) ^b	2.9 (2.2–3.6) ^c

Abbreviation: APC, activated protein C.

^a $P < .001$, in comparison with the sham operation group.

^b $P < .05$, in comparison with the sham operation group.

^c $P < .05$, in comparison with the solvent control group.

Horseradish peroxidase–coupled secondary antibodies (1:4000, Abcam 6728; Abcam 6721) were added, and then the samples were incubated at room temperature for 1 hour. After washing the membrane, chemiluminescence was applied to detect the expression of proteins NF- κ B p65 and I κ B in the cytoplasm and nuclei and proteins TNF- α and IL-1 β in the cytoplasm (Applygen Technologies Inc, Beijing, China).^{14,15} Protein bands were analyzed by Imagemag Software (Image Lab™ 4.1); the results were presented as the volume ratio of the target protein and internal control protein.

Detection of expression of inflammatory factors via enzyme-linked immunosorbent assay. Blood samples of all groups were collected via jugular venipuncture into vacutainer tubes with Lithium heparin. Detection of TNF- α and IL-1 β production in whole blood cell culture supernatants were determined by enzyme-linked immunosorbent assay system (Abcam 100785 [TNF- α]; Abcam 100768 [IL-1 β]).

Statistical Methods

The results of neurological function scoring were expressed as median and quartile. Other data were expressed as mean \pm standard error. SPSS 19.0 Software was adopted to perform nonparametric Kruskal–Wallis analysis and the Dunn multiple comparison test. Independent sample *t* test or 1-way analysis of variance and Newman–Keuls multiple comparison test were used for other intergroup comparisons. A significance level of $P < .05$ was adopted.

Results

Activated Protein C Improved Nerve Function Scoring After Focal Cerebral Ischemia–Reperfusion in Rats

The results of nerve function scoring before ischemia and at 24 and 72 hours after the start of ischemia in rats were shown in Table 1. Rats in the sham operation group performed normally in the nerve function scoring. After ischemia–reperfusion, there were significant difference in the nerve function scores among the 3 groups (KW = 41.00, $P < .001$). Compared to the sham operation group, rats in the solvent control group received significantly higher scores at 24 and 72 hours after the start

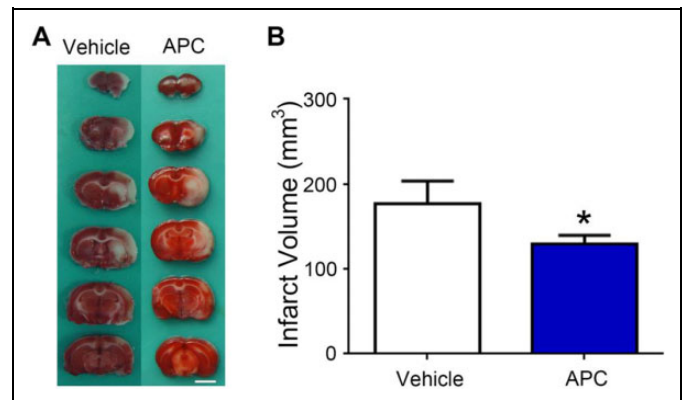


Figure 1. Effect of APC on infarct volume after focal cerebral ischemia–reperfusion in rats (A) TTC staining in rats, scale bar 5 mm; (B) infarct volume in the 2 groups, in comparison with the solvent control group, * $P < .05$, $n = 6$. (Vehicle group: normal saline was treated, 1 mL/kg; APC group: APC was treated, 2 mg/kg). APC indicates activate protein C; TTC, 2,3,5-triphenyltetrazolium chloride.

of ischemia–reperfusion ($D = -30.06$, $P < .001$; $D = -29.44$, $P < .001$); rats in APC group received significantly higher scores at 24 hours after the start of ischemia–reperfusion ($D = -22.25$, $P < .05$). There was no significant difference between the sham operation group and the APC in the scoring at 72 hours after the start of ischemia–reperfusion ($D = -11.63$, $P > .05$). In comparison with the solvent control group, there was a downtrend in the nerve function scoring in the APC group at 24 hours after start of ischemia–reperfusion, but such difference was not significant ($D = 7.813$, $P > .05$); however, the scores greatly improved after 72 hours ($D = 20.31$, $P < .05$).

Activated Protein C Reduced Infarct Volume After Focal Cerebral Ischemia–Reperfusion in Rats

The results of TTC staining of the solvent control group and APC group at 72 hours after the start of ischemia were shown in Figure 1A. The cerebral infarct volumes of the 2 groups were (177.00 ± 26.63) mm³ and (129.67 ± 10.02) mm³, respectively (Figure 1B). Activated protein C treatment can significantly reduce the infarct volume after focal cerebral ischemia–reperfusion in rats compared with the solvent control group ($t = 2.850$, $P = .013$; Figure 1).

Activated Protein C Improved BBB Permeability After Focal Cerebral Ischemia–Reperfusion in Rats

The results showed that there were significant differences among the 3 groups in BBB permeability at 72 hours after the start of ischemia (Figure 2, $F = 65.21$, $P < .001$). The level of EB in the solvent control group and APC group significantly increased ($q = 15.43$, $q = 11.84$, $P < .001$) compared to the sham operation group; in comparison to the solvent control group, the level of EB in the brain tissues in the APC group decreased significantly ($q = 3.598$, $P < .05$; Figure 2).

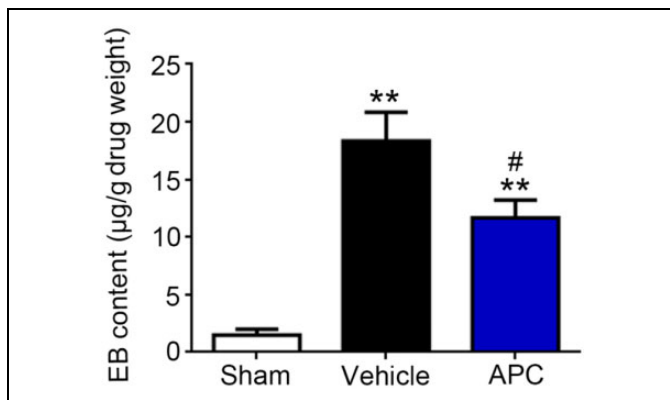


Figure 2. Effect of APC on blood–brain barrier permeability after focal cerebral ischemia–reperfusion in rats ($n = 6$, in comparison with the sham operation group, $**P < .001$; in comparison with the solvent group, $\#P < .05$. sham: placebo surgery vehicle group: normal saline was treated, 1 mL/kg; APC group: APC was treated, 2 mg/kg). APC indicates activate protein C.

Activated Protein C Reduced Expression of Inflammatory Factors After Focal Cerebral Ischemia–Reperfusion in Rats

At 72 hours after the start of ischemia, expressions of protein NF- κ B in the cytoplasm and nuclei and protein I κ B in the cytoplasm changed significantly. Compared with the sham operation group, in the solvent control group, the expression of NF- κ B decreased significantly and the expression of protein I κ B decreased in the cytoplasm, while the expression of NF- κ B increased in the nuclei. In comparison with the solvent control group, in the APC group, the expression of proteins NF- κ B and I κ B in the cytoplasm increased, while the expression of NF- κ B in the nuclei decreased. There was a significant difference in the expression between proteins TNF- α and IL-1 β in the cytoplasm (Figure 3). Compared with the sham operation group, the expression of both protein, TNF- α and IL-1 β , in the solvent group increased significantly; however, the expression of both proteins TNF- α and IL-1 β in the APC group were significantly lower than that in the solvent control group (Figure 3). In consistent with the finding above, the detection of TNF- α and IL-1 β in blood further verified that both TNF- α and IL-1 β levels were increased dramatically with the vehicle treatment. This elevation was largely blocked with APC treatment (Figure 4).

Discussion

Activated protein C can initiate signal transduction that drives multiple, diverse, and independent cellular activities, many of which are called cell-protective activities, including anti-apoptosis and anti-inflammatory activities, favorable changes of gene expression, and homeostasis of the endothelial barrier.^{16–18} Its ability to activate beneficial cellular signal transduction is currently a hot research topic.¹⁹ The neuroprotective effect of APC was first discovered in the MCAO models in rats. Treatment with APC before and immediately after ischemia

can improve cerebral blood flow and reduce infarct volume, brain edema, and neutrophil infiltration. In recent years, in vitro and in vivo studies have confirmed that APC plays an important role in direct protection of endothelial cells, stability of the BBB, protection of neurons, regeneration of nerves, and neovascularization.^{20,21}

In this study, APC significantly decreased neurological function scores and infarct volume after 72 hours of cerebral ischemia–reperfusion in rats, which suggests that APC has a neuroprotective effect in ischemic brain injury. Meanwhile, we observed the neurological function scores at 24 and 72 hours after the start of ischemia and found that the scores in the solvent control groups showed a decreasing trend over time, which may be a result of self-recovery of rats. The scoring results at 72 hours in the APC group were evidently lower than the results at 24 hours, which implies that APC has a certain level of long-term therapeutic effect.

After inflammatory responses are triggered by focal cerebral ischemia, the activated inflammatory cells clog capillaries.²² Meanwhile, the released inflammatory factors can increase microvascular permeability, resulting in the no-reflow phenomenon after reperfusion of cerebral ischemic regions and subsequent exacerbation of ischemic injury in brain tissues. At the same time, inflammatory mediators can lower the expression of cell matrix adhesion receptors on astrocytes and endothelial cells, thereby reducing the interaction of astrocytes and endothelial cells with the extracellular matrix, and increasing BBB permeability.

Some studies found that APC can prevent protein NF- κ B p65 from translocating to neuronal nuclei in cultured cortical and hippocampal neurons of rats.^{23,24} Our study also examined the expression of protein NF- κ B p65 in the cytoplasm and nuclei and expression of protein I κ B in the cytoplasm. The result indicated that protein NF- κ B p65 in the cytoplasm was translocated to the nuclei during the reperfusion after cerebral ischemia, and hydrolysis of its inhibitory protein I κ B increased and APC can inhibit the activation of NF- κ B. In addition, research confirmed that NF- κ B mediates the expression of many inflammatory mediators after cerebral ischemia (eg, TNF- α , IL-1 β , Intercellular Adhesion Molecule 1 (ICAM-1), E-Selectin, Matrix metalloproteinase (MMPs), Cyclooxygenase-2 (COX-2), and inducible NO synthase (iNOS)) and that downregulation of the transcription-promoting activity of NF- κ B can reduce focal cerebral ischemic injury.¹⁸ In this study, APC evidently inhibited the expression of proteins TNF- α and IL-1 β during the reperfusion after cerebral ischemia. Therefore, we speculate that APC may inhibit the activation and nuclear translocation of NF- κ B, thereby downregulating the transcriptional expression of inflammatory factors, reducing BBB permeability, and playing a protective role against BBB injury.

Activated protein C is a novel multitarget drug for which phase I clinical trials have been completed. Its phase II clinical trials in the treatment of acute ischemic stroke are ongoing. This study is a preliminary exploration of the effect of APC on the inflammatory responses involved in

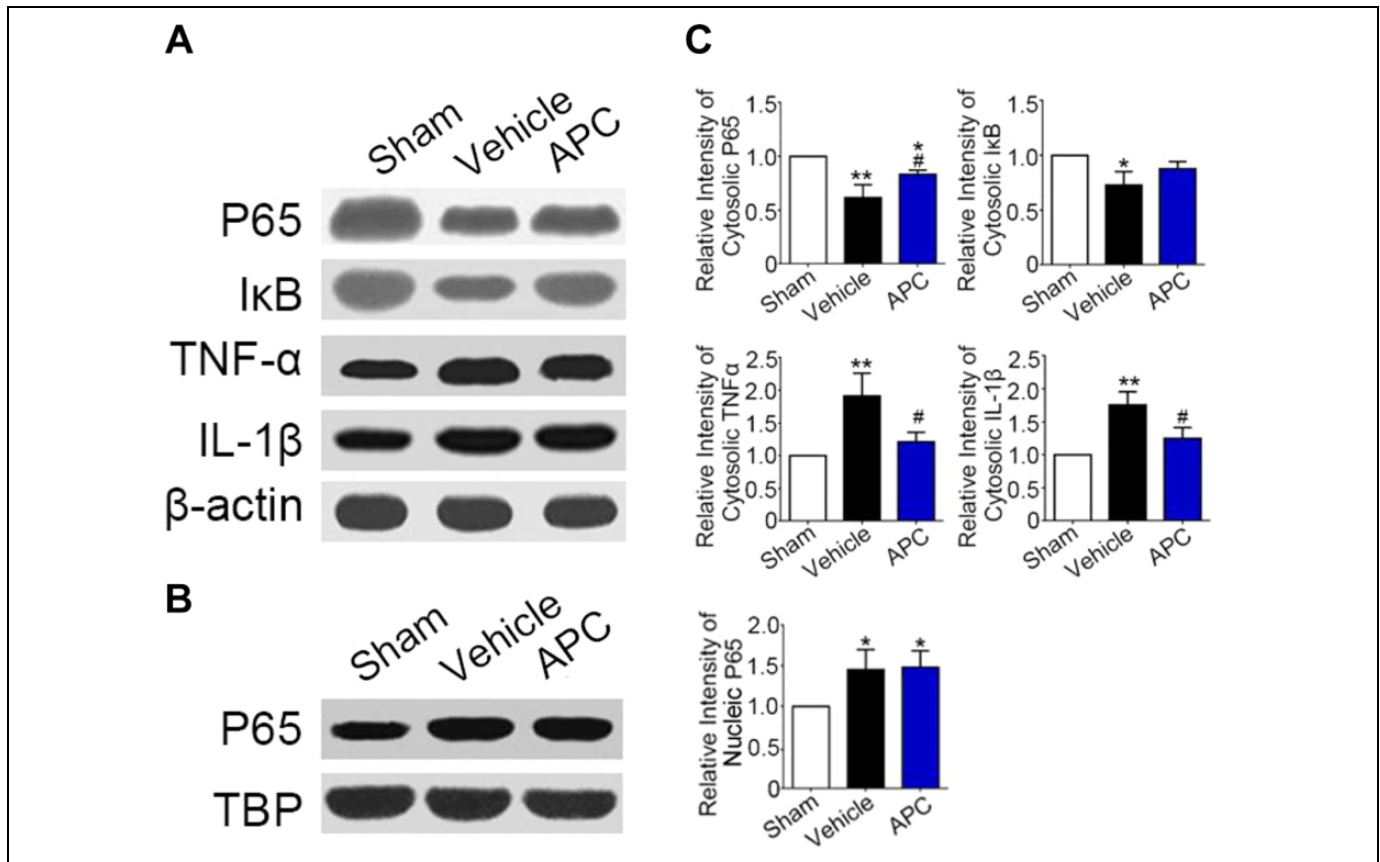


Figure 3. Effect of APC on expression of inflammatory factors after 72-hour focal cerebral ischemia–reperfusion in rats. A, Western blot of cytosolic proteins; (B) Western blot of nucleus proteins; (C) normalization of protein content to actin in each group ($n = 6$, in comparison with the sham operation group, $*P < .05$, $**P < .01$; in comparison with the solvent group, $\#P < .05$. Sham: placebo surgery, vehicle group: normal saline was treated, 1 mL/kg; APC group: APC was treated, 2 mg/kg). APC indicates activated protein C.

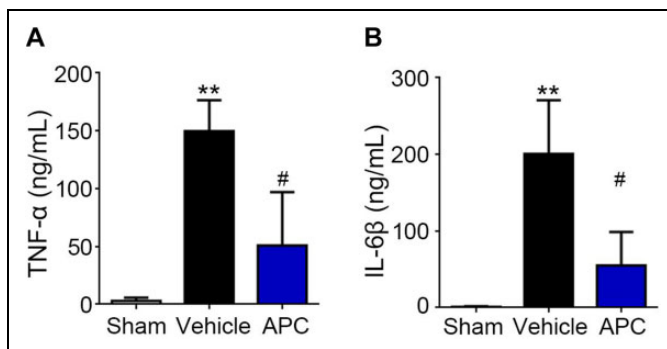


Figure 4. Enzyme-linked immunosorbent assay of TNF- α /IL-6 in the blood after focal cerebral ischemia–reperfusion in rats ($n = 6$, in comparison with the sham operation group, $**P < .01$; in comparison with the solvent group, $\#P < .05$. Vehicle group: normal saline was treated, 1 mL/kg; APC group: APC was treated, 2 mg/kg). APC indicates activated protein C; IL-6, interleukin 6; TNF- α , tumor necrosis factor.

BBB injury resulted from cerebral ischemia. However, tissue injury due to focal cerebral ischemia is triggered by a series of pathophysiological events occurring among blood capillaries–brain parenchymal cells, which eventually cause

neuron apoptosis. Therefore, neurons, cerebral capillaries, and neuroglial cells should be deemed as a functional entity. Comprehensive protection of them can therefore maintain precise balance between blood supply in the brain micro-environment and energy demand of neurons, so as to reduce neurological deficit and nerve cell apoptosis and promote the recovery of nerve function. Further research can be focused on observing the influence of APC on various components of NVU as well as their interaction to further improve research on its mechanisms.

Conclusions

In our study, we observed APC can reduce levels of inflammatory factors proteins TNF- α and IL-1 β , reduce injury to the BBB, and exert a neuroprotective effect in focal cerebral ischemia–reperfusion in rats by inhibiting the activation and nuclear translocation of NF- κ B. It may indicate a therapeutic approach for ischemic brain injury.

Authors' Note

Jinqiao Wang and Gaofeng Rao contributed equally to this work.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by Zhejiang Medical Technology Program [grant number 2018KY918].

ORCID iD

Yifan Ma  <https://orcid.org/0000-0002-9371-4365>

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