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*Original Article*

# (+)-Borneol is neuroprotective against permanent cerebral ischemia in rats by suppressing production of proinflammatory cytokines

Lei Chang[1,2,Δ], Chun-Yu Yin[1,2,Δ], Hai-Yin Wu[1,2], Bin-Bin Tian[1,2], Yan Zhu[1,2], Chun-Xia Luo[1,2], Dong-Ya Zhu[1,2,3,✉]

[1] *Institution of Stem Cells and Neuroregeneration, and* [2] *Department of Pharmacology, School of Pharmacy, Nanjing Medical University, Nanjing, Jiangsu 211166, China;*
[3] *The Key Laboratory of Human Functional Genomics of Jiangsu Province, Nanjing, Jiangsu 211166, China.*

**Abstract**

Stroke is one of the leading causes of disability and death globally. It occurs when a major artery is occluded in the brain and leads to death of cells within the injured tissue. (+)-Borneol, a simple bicyclic monoterpene extracted from traditional Chinese medicine, is widely used in various types of diseases. However, no study has proved the effects of (+)-borneol on functional recovery from permanent ischemic stroke and the mechanism is still unknown. Here, we report that in the rat model of permanent cerebral ischemia, we found that (+)-borneol (1.0 mg/kg) significantly ameliorated infarct size and neurological scores *via* reducing the expression of inducible nitric oxide synthase (iNOS) and tumor necrosis factor-alpha (TNF-α) in a dose dependent manner. Notably, (+)-borneol showed long-term effects on the improvement of sensorimotor functions in the photothrombotic model of stroke, which decreased the number of foot faults in the grid-walking task and forelimb asymmetry scores in the cylinder task, at least in part through reducing loss of dendritic spines in the length, brunch number and density. These findings suggest that (+)-borneol could serve as a therapeutic target for ischemic stroke.

**Keywords:** (+)-borneol, neuroprotective effects, permanent cerebral ischemia, anti-inflammation, functional recovery, dendritic spines

## Introduction

Stroke is a leading cause of morbidity, mortality, and long-term disability worldwide, which represents a major public health problem[1]. Excitotoxicity, peri-infarct depolarizations, inflammation and apoptosis are induced following cerebral ischemia[2]. Studies in recent years have focused on effective therapy to improve the damaged sensory, motor, and cognitive functions. However, because of the complex pathogenesis of ischemic stroke and difficulty in drug delivery across the blood-brain barrier (BBB), there are few effective therapies for stroke patients. Therefore, it is urgent to find out the most effective treatment for stroke.

Inflammation participates in secondary damage after cerebral ischemia. Post-ischemic inflammation immediately starts in the intravascular compartment after artery occlusion as triggering the coagulation cascade

[Δ]These authors contributed equally.

[✉]Corresponding author: Dong-Ya Zhu, Ph.D., Departments of Pharmacology, School of Pharmacy, Nanjing Medical University, Nanjing, Jiangsu 211166, China, Tel/Fax: 86-25-86868469/86-25-86868469, E-mail: dyzhu@njmu.edu.cn.

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*via* the ensuing hypoxia, changes in shear stress, and production of reactive oxygen species (ROS), as well as the local production of pro-inflammatory molecules; thereby, it contributes to the expansion of brain damage and tissue injury[3]. Thus, it is reasonable that the inhibition of inflammatory responses may in part be attributed to neuroprotection at a subsequent ischemic stage.

(+)-Borneol ($C_{10}H_{18}O$) is a simple bicyclic monoterpene extracted from traditional Chinese medicine. Researchers have studied many aspects of bioactivities of (+)-borneol, including improvement of the energy metabolism[4], anti-inflammatory effect[5], and antiepileptogenic effect[6]. Especially, (+)-borneol obviously loosens the intercellular tight junction in the blood-brain barrier (BBB) and enhance the distribution of drugs in the brain tissue, as it could increase number and volume of pinocytotic vesicles in BBB cells and then promote the transportation of substance by cell pinocytosis[7]. Recently, it has been reported that, as a chemosensitizer, (+)-borneol has synergistic apoptosis-inducing effects with curcumin[8]. Moreover, our previous study found that (+)-borneol ameliorates mechanical hyperalgesia by enhancing $GABA_A$ receptor-mediated GABAergic transmission in the spinal cord without producing motor deficit[9]. The neuroprotective effect of (+)-borneol was addressed just in an *in vitro* ischemic model of oxygen-glucose deprivation followed by reperfusion (OGD/R)[4]. However, little is known about the *in vivo* effects of (+)-borneol on functional recovery following ischemic stroke. Because the most common type of stroke in humans is permanent occlusion of the cerebral arteries, we decided to investigate the role of (+)-borneol on functional recovery after permanent ischemic stroke by *in vivo* models and possible mechanisms underlying the protective effects.

In this study, we examined the effect of (+)-borneol in the acute phase of permanent cerebral ischemia. Our results indicated that (+)-borneol improves stroke outcome probably through decreasing the expression of proinflammatory cytokines. Meanwhile, we investigated the long-term effects of (+)-borneol following the photothrombotic model of stroke and found that (+)-borneol significantly improved sensorimotor functions by ameliorating ischemia-induced loss of dendrite spines.

## Materials and methods

### Drugs

(+)-Borneol was supplied by Simcere Pharmaceutical Group. All cell culture medium and supplements were purchased from Gibco (Grand Island, NY, USA). 2,3,5-tripenyltetrazolium chloride (TTC) and Rose Bengal were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Animals

The experimental protocol was approved by the Institutional Animal Care and Committee of Nanjing Medical University. In this study, adult male Sprague-Dawley rats (240-270 g) and C57BL/6J mice (22-27 g) were purchased from Shanghai B&K Universal Group Limited. The animals were housed 22±2°C on a 12-hour light/dark cycle with access to food and water ad libitum. Under the randomization schedule, an experimenter labeled all animals before allocation. Experiments were performed by investigators who were unknown to group allocation.

### Surgical preparation

Transient middle cerebral artery occlusion (tMCAO) was performed to induce focal cerebral ischemia as described previously[10]. Anesthesia was induced in rats with 4% isoflurane and was maintained with 2% isoflurane in a gas mixture of 5% $CO_2$ and 95% $O_2$ using a facemask. A 4/0 surgical nylon monofilament with rounded tip was introduced into the left internal carotid artery through the external carotid stump, advanced 20-21 mm past the carotid bifurcation until slight resistance was left. The middle cerebral artery was effectively occluded. Then, the filament was withdrawn after 120 min and the external carotid artery was ligated. During the process, body temperature was maintained at 37±0.5°C. In sham-operated rats, the occluding filament was inserted only 7 mm above the bifurcation. Animals were returned to their cages and monitored until they recovered from anesthesia.

Permanent middle cerebral artery occlusion (pMCAO) was performed to induce focal cerebral ischemia as described previously. Anesthesia was induced in rats with 4% isoflurane and was maintained with 2% isoflurane in a gas mixture of 5% $CO_2$ and 95% $O_2$ using a facemask. A 4/0 surgical nylon monofilament with rounded tip was introduced into the left internal carotid artery through the external carotid stump, advanced 20-21 mm past the carotid bifurcation until slight resistance was left. The middle cerebral artery was effectively occluded. Then, the filament was sutured. During the process, body temperature was maintained at 37±0.5°C. In sham-operated rats, the occluding filament was inserted only 7 mm above the bifurcation. Animals were returned to their cages and monitored until they recovered from anesthesia.

Photothrombotic model of focal stroke: A photothrombotic lesion was induced as described pre-

viously[11]. Male C57BL/6J mice (22-27 g) were anesthetized with 4% isoflurane for induction and was maintained with 2% isoflurane in a gas mixture of 5% $CO_2$ and 95% $O_2$ using a facemask. The animals were placed in a stereotaxic frame and body temperature was maintained at 37°C. The skin above the skull was incised and an optic fiber bundle (aperture: 1.0 mm) mounted on a cold light source (Schott KL 1500) was positioned 2 mm lateral to the midline and 1 mm posterior to the bregma. Next, 100 μL Rose Bengal (10 mg/ml in 0.9% NaCl) was injected intravenously. After 5 min, the illumination period of 15 min was started. The skin was sutured and the animals recovered in their cage.

**Neuroscore assessment**

Neuroscore assessment was performed by an experimenter blinded to the experimental groups[12](rating scale: 0 = no deficit, 1 = failure to extend left forepaw, 2 = decreased grip strength of left forepaw, 3 = circling to left by pulling the tail, and 4 = spontaneous circling).

**Determination of infarct size**

The infarct size was determined as described previously[13]. Briefly, brains were removed rapidly and frozen at –20°C for 5 minutes. Coronal slices were made at 1-2 mm from the frontal tips and sections were immersed in 2% TTC at 37°C for 20 minutes. Infarct volume was expressed as expressed as a percentage area of the coronal section in the infarcted hemisphere.

**Grid-walking task**

The grid-walking task apparatus was manufactured as previously described[11]. Mice ran one trial per day at approximately the same time each day. The grid area was 32 cm × 20 cm × 50 cm (length × width × height) with a 12 mm square wire mesh. Behavior was recorded using a camera that was placed underneath the grid, in order to assess the animals' stepping errors (i.e. 'foot faults'). Animals were given 5 min to walk atop the elevated wire surface. Foot faults for each limb were counted and compared to the overall step number made by that limb. Thus, % foot faults was calculated by: number of foot faults / (foot faults + number of non-foot-fault steps) × 100%. A step was considered a foot fault if it did not provide support and the foot went through the grid hole.

**Cylinder task**

In brief, the spontaneous forelimb task encourages the use of forelimbs for vertical wall exploration/press in a cylinder. When placed in a cylinder, the animal rears to a standing position, while supporting its weight with either one or both of its forelimbs on the side of the cylinder wall. Mice were placed inside a Plexiglas cylinder (30 cm in height with a diameter of 20 cm) and videotaped for 5 min. Videotaped footage of animals in the cylinder was evaluated quantitatively to determine forelimb preference during vertical exploratory movements. While the video footage was played in slow motion (one-fifth real-time speed), the time (in seconds) during each rear that each animal spent on the right forelimb, the left forelimb, or both forelimbs was calculated. Only rears in which both forelimbs could be clearly seen were timed. The percentage of time spent on each limb was calculated, and these data were used to derive an asymmetry index as follows: (percentage of ipsilateral use)-(percentage of contralateral use).

**Western blot analysis**

Western blot analysis was performed as described previously[13]. The primary antibodies were as follows: rabbit anti-iNOS (1:1000, Alexis), rabbit anti-TNF-α (1:500, Abcam). Appropriate horseradish peroxidase-linked secondary antibodies were used for detection by enhanced chemiluminescence (Pierce, Rocdford, IL, USA).

**Microglial cell culture**

The immortalized murine microglia cell line, BV-2[14], was purchased from the Cell Bank, Chinese Academy of Sciences (CBP60603). BV-2 cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Invitrogen Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained in a humidified incubator at 37°C in 5% $CO_2$.

**Golgi-Cox staining**

Golgi-Cox staining was used to show subtle morphological alterations in neuronal dendrites and dendritic spines. For morphological analysis, 5 random neurons from each sample were measured, and the average was regarded as the final value of one sample.

**Statistical analysis**

Comparisons among multiple groups were made with one-way ANOVA (one factor) or two-way ANOVA (two factors) followed by Scheffé's post hoc test. Comparisons between two groups were made with a two-tailed Student's *t*-test. Data were presented as the

mean±S.E.M., and $P < 0.05$ was considered statistically significant.

## Results

### (+)-Borneol reverses tMCAO-induced focal cerebral ischemia

To determine whether (+)-borneol has neuropreotective effect after ischemic stroke, we randomly allocated 105 Sprague-Dawley rats to 7 experimental groups ($n$ = 15 for each group), including the sham group, the vehicle-treated group and the (+)-borneol-treated groups (0.25, 0.5, 1.0, 2.0, and 4.0 mg/kg). We subjected rats to tMCAO and treated them with drugs by tail intravenous injection at 2 hours after reperfusion. Neurologic scores were assessed at 48 hours after reperfusion. The sham group and the vehicle-treated group were injected with vehicle. As shown in ***Fig. 1A*** and ***B***, ischemia significantly increased infarct size and neurological scores, which were reversed by (+)-borneol on dosages from 0.5 mg/kg to 2.0 mg/kg.

### (+)-Borneol contributes to neuroprotection and reduces pro-inflammatory cytokine expression in the acute phase of focal permanent cerebral ischemia

It has been reported that permanent arterial occlusion is likely to be more relevant to the majority of ischemic stroke cases in humans[15]. To mimic a typical human stroke, which does not undergo reperfusion, we used a permanent MCAO in this study. For investigating the role of (+)-borneol in permanent cerebral ischemia, 45 male Sprague-Dawley rats were randomly divided into the sham group, the vehicle-treated group and the (+)-borneol-treated groups (1.0 mg/kg), with 15 rats in each group. The terminal half-life ($t_{1/2}$) of borneol was 2 h following cerebral ischemia-reperfusion[16]. We subjected the rats to pMCAO and administered drugs by tail intravenous injection 2 hours and 5 hours after pMCAO, respectively. The sham group and the vehicle-treated group were injected with vehicle administration. Neurologic scores were assessed at 48 hours after reperfusion. The cerebral infarction was found mostly in the cerebral cortex in the vehicle-treated group. A dramatic decrease in infarct size was shown in the (+)-borneol-treated group at 1.0 mg/kg ($F_{5,62}$ = 126.60, $P$ = 0.012), compared to the vehicle-treated group (***Fig. 2A***). Furthermore, the neurological score was measured 48 h after permanent cerebral ischemia. The score was significantly lower in the (+)-borneol-treated group than the vehicle-treated group (***Fig. 2B***). To determine the anti-neuroinflammatory effects of (+)-borneol *in vivo*, we measured the expression of iNOS and TNF-α in the rat ischemic cortex after 24 h permanent ischemic stroke. As shown in ***Fig. 2C*** and ***D***, (+)-borneol markedly downregulated iNOS and TNF-α protein levels in the ischemic region of the rat cortex induced by pMCAO.

Taken together, our results demonstrated the neuroprotective effect of (+)-borneol probably *via* reducing the expression of pro-inflammatory cytokines in a rat model of permanent cerebral ischemia.

### (+)-Borneol improves functional recovery after permanent ischemic stroke

The impairment of sensorimotor functions is the most typical and dominant clinical problem, which is induced by focal cerebral ischemia. Many patients with small infarcts survive after ischemic stroke, suffering from motor impairments of the contralateral upper limb as the most common functional deficit following cerebral ischemia. Owing to the difficulties of pMCAO in reproducibility and mortality and leading to damage beyond MCA territory and corresponding associated deficits by many other arteries probably being blocked[17], we used a photothrombosis stroke model of focal stroke, the permanent nature of this stroke

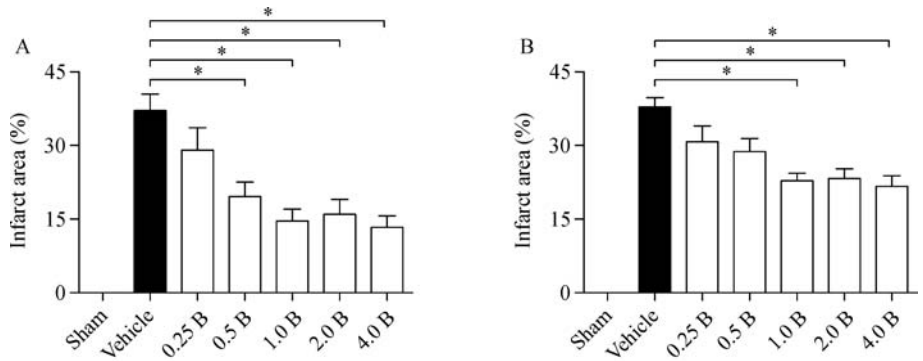


***Fig. 1*** **(+)-Borneol has neuroprotective effects in acute phase of focal cerebral ischemia with reperfusion.** A-B: Rats were subjected to tMCAO. Drugs were immediately given (i.v.) after reperfusion. Infarct size (A) and neurological score (B) were measured at 48 hours after reperfusion. B: borneol, 0.25B-4.0B: 0.25-4.0 mg/kg borneol. Data are mean ± S.E.M. (n = 13-15, *$P$ < 0.05, *vs*. vehicle). tMCAO: transient middle cerebral artery occlusion.

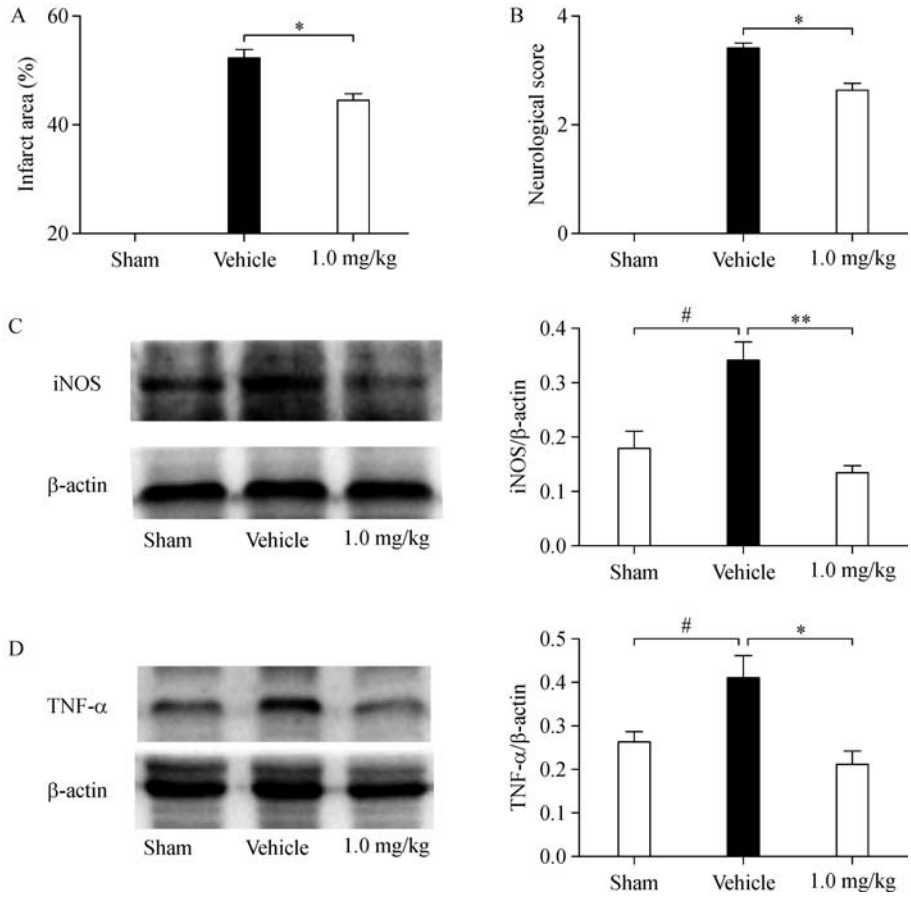


***Fig. 2*** **(+)-Borneol contributes to neuroprotection and reduces pro-inflammatory cytokines expression in acute phase of focal permanent cerebral ischemia.** A-D: Rats were subjected to pMCAO. Drug or vehicle was given (i.v.) 2 hours and 5 hours after pMCAO, respectively. Infarct area (A) and neurological score (B) were measured at 48 hours after pMCAO. Data are mean ± S.E.M. (n = 13-15, **P* < 0.05, *vs*. vehicle). C-D: Immunoblots showing iNOS (C) and TNF-α (D) levels in the cortex ischemic penumbra after 24-hour-pMCAO. Data are mean ± S.E.M. (n = 6-7, #*P* < 0.05, *vs*. Sham ; ***P* < 0. 01, **P* < 0.05, *vs*. vehicle). pMCAO: permanent middle cerebral artery occlusion.

model, to investigate the link between the development of motor cortex ischemia and motor deficits[18]. Forty-five C57BL/6J mice (22 g- 27 g) were randomly allocated to 3 experimental groups (*n* = 15 for each group), including the sham group, the vehicle-treated group and the (+)-borneol-treated groups. The mice were subjected to photothrombosis stroke and administered with drugs by tail intravenous injection for consecutive 14 days (0.75 mg/kg/d). Sensorimotor functions were assessed at day 14, 28 and 42 after photothrombosis stroke (***Fig. 3A***). In this study, the grid-walking task was conducted to measure the hindlimb foot faults and the cylinder task was used to assess the spontaneous forelimb preference for rearing in cylinder. As shown in ***Fig. 3B*** and ***C,*** Forty-five permanent cerebral ischemia significantly increased the number of foot faults in the grid-walking task and forelimb asymmetry scores in the cylinder task, compared with the sham group, indicating substantially injured sensorimotor functions. On the contrary, treatment with (+)-borneol prominently ameliorated ischemia-induced impairments of sensorimotor functions, compared to vehicle-treated animals. These results suggest that (+)-borneol promotes functional recovery from permanent stroke with a long-term effect.

### (+)-Borneol rescues ischemia-induced dendritic spines loss

Dendritic spines are protrusions from the main shaft of neuronal dendrites, which receive most of the excitatory synaptic input in the cerebral cortex[19]. The deleterious effect of cerebral ischemia on dendritic spine morphology and function has been associated with impaired synaptic transmission and neurological deterioration[20].We thus investigated whether (+)-borneol produces the long-term effect of functional recovery from permanent ischemic injury *via* influencing dendritic spines. Mice then were sacrificed to measure dendrite spine length, brunch number and density in peri-infarct region at day 43 (***Fig. 4A***). The permanent ischemia caused substantial decreases in dendrite spine length, brunch number and density of neurons in the

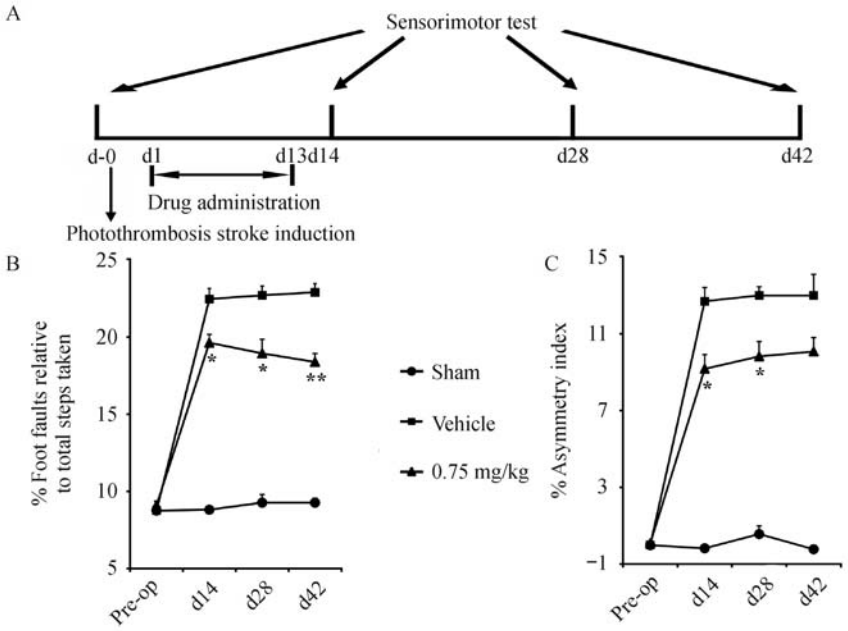


***Fig. 3*** **(+)-Borneol exhibits a long-term effect on experimental permanent ischemic stroke for functional recovery.** A: Schematic representation of experimental design for B–C. B: Analysis of forelimb foot faults on grid-walking task and (C) assessment of forelimb asymmetry using the cylinder task at indicated times after photothrombosis stroke. Data are the mean±S.E.M., (PT: photothrombosis stroke, $n$ = 15, $^{**}P<0.01$, $^{*}P<0.05$, *vs.* vehicle).

striatum and the motor cortex. Treatment with (+)-borneol prevented from ischemia-induced alterations of dendrites (***Fig. 4B-4D***). Our results suggest that (+)-borneol facilitates the recovery of neurological function by reducing loss of dendritic spines after permanent ischemic stroke.

### (+)-Borneol decreases the level of proinflammatory cytokines in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells

Inflammatory response plays a key pathogenic role in the secondary injury following ischemic stroke[21]. Activated microglia contributes to ischemic brain injury *via* the production of inflammatory cytokines[22]. To examine whether (+)-borneol inhibits inflammation and thereby alleviates cerebral ischemic injury, we pre-treated BV-2 cells with different concentrations of (+)-borneol (0.01, 0.1, 1.0, 10.0 nmol/L) for 30 min and then treated with LPS (2μg/mL) for 24 hours. Subsequently, we examined the protein levels of iNOS and TNF-α by western blot analysis. As showed in ***Fig. 5A*** and ***B***, LPS dramatically increased levels of iNOS and TNF-α, and (+)-borneol significantly reduced the protein expression of iNOS and TNF-α in a dose-dependent manner. Collectively, the reduction of inflammatory factors may explain the neuroprotective effect of (+)-borneol against cerebral ischemic injury.

## Discussion

Ischemic stroke induces cerebral injury and the disruption of BBB[23]. Currently, stroke therapies are designed to establish early reperfusion by thrombolytic and/or mechanical recanalization of obstructed blood vessels. Due to the fact that the restoration of blood flow may lead to hemorrhagic transformation or massive brain edema[24], there is an unmet need for the treatment of ischemic stroke. Here, we demonstrated that (+)-borneol can serve as a potential medicine for the treatment of stroke. Our study here showed that (+)-borneol has substantial neuroprotection in permanent MCAO model of stroke *via* inhibiting the expression of proinflammatory cytokines.

Dendritic spines play a fundamental role in excitatory synaptic transmission, considering as the "hot spot" of synaptic plasticity[25–26]. The rearrangement of the structures and functions of most spines influences synaptic connectivity and neuronal plasticity, which could control learning, memory, behavior and motor coordination[27]. In our study, we chose the photothrombosis stroke model to investigate the long-term beneficial effect of borneol on sensorimotor functions, which causes the occlusion of microvasculature in a targeted region of cortex with the accompanying mitochondrial dysfunction, inflammation, neurodegeneration and behavioral deficits[28]. We found that focal

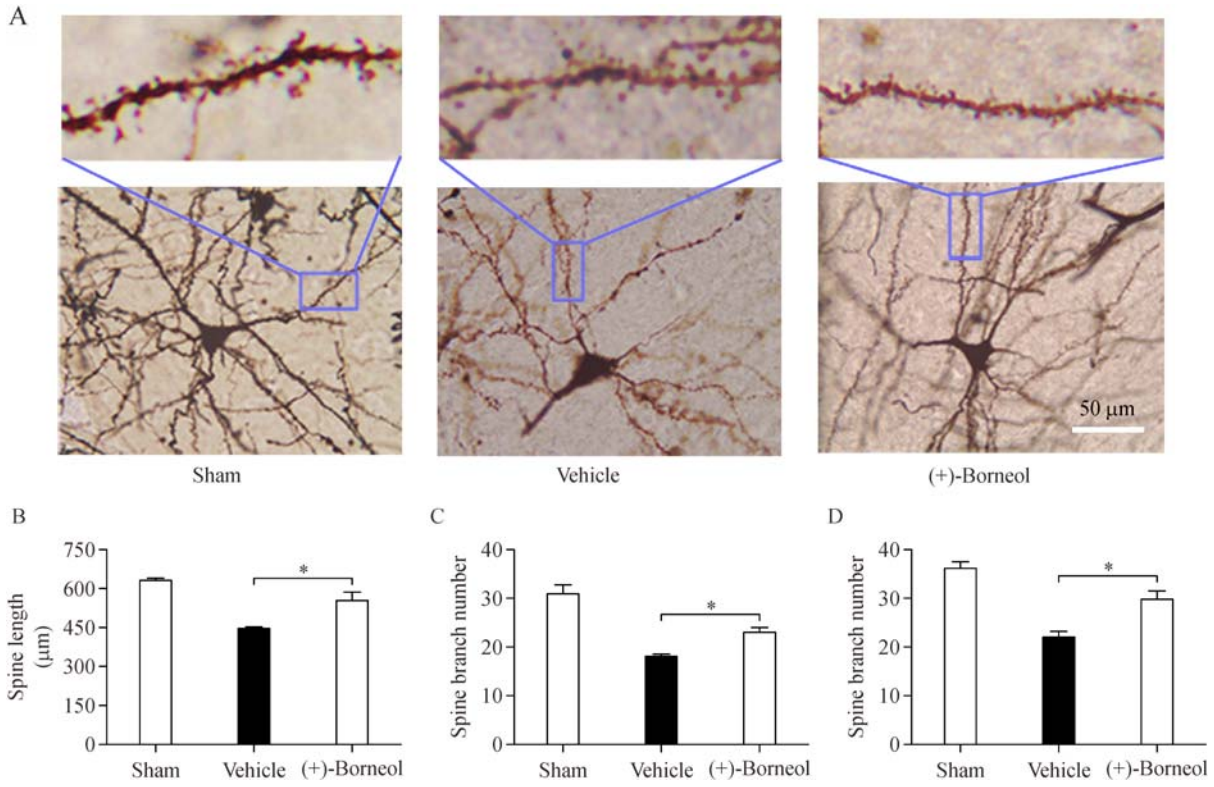


***Fig. 4*** **(+)-Borneol reduces dendritic spine loss following ischemic injury.** Representative images (A) and bar graphs showing dendrite spine length (B), brunch number (C) and density of spines in the peri-infarct cortex in the mice subjected to photothrombotic stroke at day 43. The mice were treated by (+)-borneol (0.75 mg/kg/d, i.v.) or vehicle for consecutive 14 days beginning at 2 hours after stroke. Five neurons were measured in each biological sample. Data are the mean±S.E.M., (*$P < 0.05$, *vs.* vehicle).

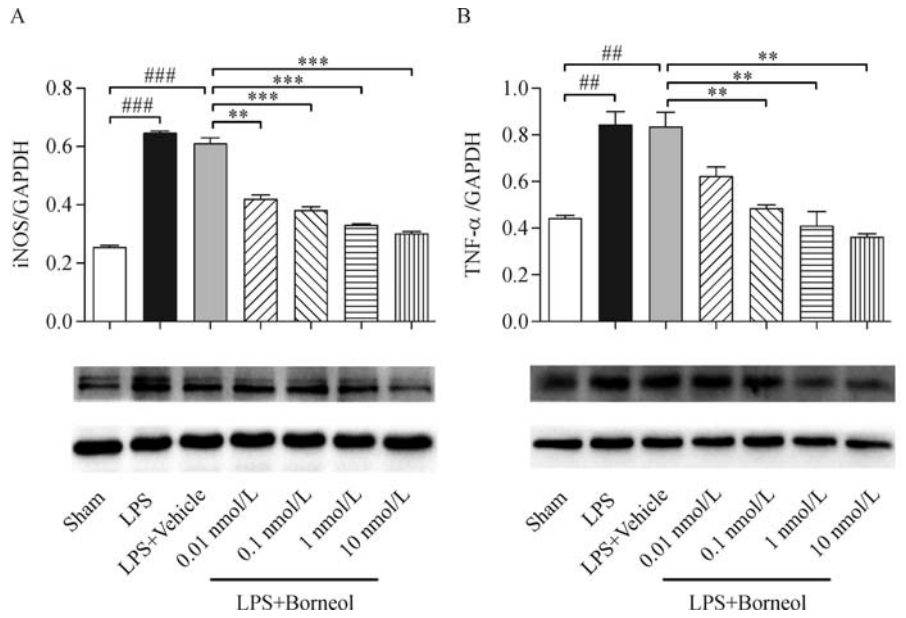


***Fig. 5*** **(+)-Borneol downregulates the expession of proinflammatory cytokines in LPS-activated BV2 Microglial Cells.** A-B: Immunoblots showing iNOS (A) and TNF-α (B) levels in the BV2 microglial cells pretreated with different concentrations of (+)-borneol (0.01, 0.1, 1.0, 10.0 nmol/L) for 30 minutes following by stimulation with LPS (2 μg/mL; for 24 hours). Data are the mean±S.E.M., $n = 3$, ($^{###}P < 0.001$, $^{##}P < 0.01$, compared to the sham group; ***$P < 0.001$, **$P < 0.01$, compared to the vehicle group).

cerebral ischemia induced a severe loss of dendritic spines in peri-infarct regions. It may be attributed to the production of reactive oxygen species, the increased glutamate level and the excessive $Ca^{2+}$ influx which cause mitochondrial damage[26,29–30]. Consequently, the extensive changes in dendritic spines may significantly affect neuronal survival[31]. Interestingly, we found that (+)-borneol dramatically reduces dendritic spines loss after stroke, we observed that (+)-borneol improves motor dysfunctions in the grid-walking task and cylinder task. Thus, our findings suggest that (+)-borneol may produce long-term beneficial effect on sensorimotor functions in the photothrombosis model of stroke by ameliorating ischemia-induced degeneration of dendrites.

Post-ischemic inflammation may have impact on the extent of tissue damage, infarct demarcation, tissue repair and functional recovery[32] .Many anti-inflammatory agents are used to prevent ischemia-induced damage in experimental models of stroke. Microglia are the resident immune cells in the central nervous system. Once activated, microglia releases pro-inflammatory molecules such as iNOS and TNF-α, and these molecules causes neuronal death and then contributes to the development of neurodegenerative diseases[33]. In our study, *in vivo*, we found that (+)-borneol decreased the expression of iNOS and TNF-α in ischemic regions of the rat cortex induce by pMCAO. *In vitro*, we examined the effect of (+)-borneol on pro-inflammatory molecules in LPS-activated BV-2 microglial cells, a model of neuroinflammation. Obviously, (+)-borneol significantly inhibited the expression of iNOS and TNF-α in a dose-dependent manner in the LPS-stimulated BV-2 cells, indicating an anti-inflammatory effect of (+)-borneol by inhibiting the expression of proinflammatory cytokines. In addition, microglia cells express iNOS to increase the production of nitric oxide (NO) under neuroinflammatory[34]. The overproduction of superoxide and NO can trigger brain damage after ischemic stroke. It is possible that (+)-borneol could also inhibit the production of NO, thereby promoting neuronal survival.

Little is known about the effect of (+)-borneol on cerebral ischemia, although some have proved that (+)-borneol has synergistic effect with other drugs following ischemia[7,35] and (+)-borneol has a neuroprotective effect against OGD[4]. Here, we investigated the neuroprotective effects of (+)-borneol for the first time in both the acute phase and delayed phase by two models of permanent cerebral ischemia *in vivo*. To our knowledge, this is the first study to demonstrate that (+)-borneol has a potent protective effect against ischemia-induced motor dysfunction. (+)-Borneol not only suppresses the expression of proinflammatory cytokines but also reduces the dendritic spines loss. In addition, evidence has suggested that GABA-mediated neuronal inhibition could be an important factor in the process of delayed neuronal degeneration following cerebral ischemia[36]. What is more, the spiny neurons in central nervous system are mostly glutamatergic or GABA-nergic. Meanwhile, previous study showed that (+)-borneol directly enhances GABA activity at recombinant human $GABA_A$ receptors[37]. Thus, we cannot exclude the possibility that (+)-borneol produces neuroprotective effects by activating GABA receptors after cerebral ischemia.

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