Regulation of iNOS -NF-kappa B- COX-2 inflammatory pathway by alpha-pinene neuroprotective effects in brain ischemia model

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Abstract

Objectives Cerebral ischemia-reperfusion leads to brain tissue injury. Inflammation and apoptosis play pivotal roles in the pathology. α-Pinene is an organic compound of many aromatic plants and is known as a potent agent to possess antioxidant, and anti-inflammatory properties. Here, we sought to identify the anti-inflammatory and anti-apoptosis mechanism by which α-Pinene improves brain ischemia injury.

Methods Male Wistar rats underwent MCAO surgery for 1 hour and different doses of alpha-pinene (25, 50, and 100 mg/kg) were intraperitoneally injected immediately after reperfusion to test this hypothesis. IV, NDS, gene and protein expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), nuclear factor kappa B (NF-κB) p65, and caspase-3 were assessed 24h after reperfusion.

Key findings Results demonstrated that NF-κB p65, iNOS, and COX-2 gene and protein expression increased in the hippocampus, cortex, and striatum after 24 h of reperfusion, and alpha-pinene significantly inhibited NF-kB p65, iNOS, and COX-2 expression. Also, alpha-pinene significantly reduced the ischemia/reperfusion-induced caspase-3 activation in CA1 area of hippocampus.

Conclusions Results showed that alpha-pinene protects the cerebral against ischemic damage caused by MCAO, and this effect may be through the regulating iNOS -NF-kappa B- COX-2 and caspas-3 inflammatory and apoptotic pathways.

Introduction

Stroke is the leading cause of disabilities and cognitive deficits, and the second cause of death worldwide [1-2]. Stroke occurs when a blood vessel(s) is interrupted by a blood clot/thrombus or when blood vessel(s) rupture (i.e., hemorrhage) that's why the brain is one of the most high-energy consuming organs, the lack of oxygen and nutrient supply elicited by stroke can cause severe brain damage resulting in neurological disorders [3]. To date, only a single drug has received US Food and Drug Administration (FDA) approval for acute ischemic stroke treatment, recombinant tissue plasminogen activator (rt-PA). While rt-PA therapy restores perfusion to ischemic brain, considerable tissue damage occurs when cerebral blood flow is re-established after cerebral ischemia. Therefore, there is a critical need for novel therapeutic approaches that can “rescue” salvageable brain tissue during ischemic stroke [4-5].

Ischemic stroke is a complex disease, many molecular signaling cascades are involved solely or in combination in this disorder that besides neuronal cell loss, damage to and loss of astrocytes, as well as injury to white matter, contributes also to cerebral injury. Those molecular mechanisms include energy depletion, dissipation of ion gradients, calcium overload, excitotoxicity, oxidative stress, inflammatory response, and accumulation of ions and fluid [5-6]. Inflammation is a key origin in the pathogenic mechanisms related to cerebral ischemia [7-8-9].
Furthermore, nuclear factor (NF)-κB, which is a key downstream factor of the inflammation signaling pathway, is activated after brain ischemia, to promote inflammatory reactions and produce inflammatory molecules that further aggravate ischemic brain injury. (NF)-κB signaling is a promising therapeutic target for the treatment of ischemic stroke because the NF-κB expression reduction and its downstream signaling pathway, ultimately leads to the attenuation of ischemic brain injury. Despite extensive studies, however, the various basic inflammatory factors and free radical generators signaling pathway have not been characterized. It has been specified cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) are two well-known free radical generators. Studies have demonstrated that inhibition of NF-κB has beneficial effects on cerebral ischemia. NF-κB is the main regulator of pro-inflammatory mediators, such as iNOS and COX-2. Previous studies have shown that iNOS protein levels increased as early as 1 hour after stroke, maintained high levels for up to 24 hours and also it has been revealed that cyclooxygenase-2 (COX-2) is induced in the cortex and hippocampal structures after ischemia and it is implicated in ischemic neuronal death. Several brain regions, including the cortex, hippocampus, and striatum, are involved in ischemic injury.

Apoptosis is one of the main mechanisms of cell death in many brain diseases, including cerebral ischemia. Apoptosis increases the permeability of the mitochondrial outer membrane and causes the transfer of cytochrome c to the cytosol and leads to the formation of apoptosome. The apoptosome activates caspase 9, which is followed by the activation of caspase-3. The activation of caspase-3 induces apoptotic neuronal cell death. Therefore, inhibition of caspase-3 can significantly decrease ischemia-induced cell death. Increased activation of caspase-3 means irreversible apoptosis. Apoptotic signals induce the cleavage of caspase-3 and activated caspase-3 may initiate the caspase cascade reaction. Inflammation and apoptosis have a reciprocal relationship in ischemia/reperfusion injury.

The hippocampus plays an important role in both learning and memory. The neurons of the CA1 hippocampus are very vulnerable after cerebral ischemia. It has been shown that in ischemic stroke, apoptosis can decrease the proapoptotic proteins (caspase-3, caspase-9, and Bax) expression. Several investigations have focused on traditional Chinese medicine's therapeutic benefits in cerebral ischemia. Especially plant-derived natural medicines have multiple beneficial effects, including antioxidative, anti-inflammatory, antiapoptotic, suggesting that may improve pharmacotherapy of cerebral ischemia.

The molecular formula of plant phytochemical, alpha-pinene is C10H16 (Figure 1)

Which is of well-known representative of the monoterpenes group, and are found in pine essential oils. A wide range of its pharmacological activities have been reported, including antibiotic resistance modulation, anticoagulant, antitumor, antimicrobial antioxidant, anti-inflammatory effects.
The anti-inflammatory and antioxidant activity of alpha-pinene has been investigated in previous studies owing to the inhibition of the NF-κB. Studies have demonstrated that inhibition of NF-κB has beneficial effects on cerebral ischemia. NF-κB is the main regulator of pro-inflammatory mediators, such as iNOS and COX-2. Previous research has shown that Alpha-pinene as a compound organic attenuates ROS-mediated oxidative damage and NO production. It has been illustrated that alpha-pinene has an inhibitory effect on inflammation including inhibition of NF-kB, LTB4, and IL-1β. According to previous studies, alpha-pinene reduces cerebral edema and oxidative stress caused by cerebral ischemia.

Therefore, this study aimed to investigate the effect of alpha-pinene in a rat model of cerebral ischemia with hypothesized that Alpha-pinene protects against ischemic stroke via regulation of inflammatory and apoptosis response. Since the iNOS NF-κB, Cyclooxygenase-2 and caspase-3 are critical mediated inflammation and apoptosis after stroke, we evaluated this cell signaling pathway that is involved in stroke.

Methods

Chemicals

Alpha-pinene and TTC (2, 3, 5-triphenyltetrazolium chloride) were obtained from Sigma-Aldrich Company (Saint Louis, MO, United States). Alpha-pinene was prepared by dissolving it in Tween-80 and physiological saline.

Animals

Kerman University approved all experimental protocols of Medical Sciences, Kerman, Iran and all laboratory animals were carried out with specific consideration of EU directive 2010/63/EU. Male Wistar rats weighing 250-350 grams were used in this research. The rats were kept in standard metal cages under controlled conditions, including a 12:12-hour light: dark cycle at 22±2º C. Body temperature and vital signs were monitored. The rats were randomly divided into five groups (n=6): sham group, control (MCAO) group, 25 mg/kg α-pinene group, 50 mg/kg α-pinene group, and 100 mg/kg α-pinene group. The animals underwent surgery without the obstruction of their middle cerebral artery (MCA) in the sham group. In the control group, the cerebral ischemia was induced by MCA occlusion (MCAO) and performed as explained in detail formerly, and this group received equivalent volumes of (%10 tween 80 + %80 normal saline). Animals in groups 3 to 5 were exposed to MCAO, and then α-pinene was administered by intraperitoneal (i.p.) injection at the beginning of reperfusion. At 24 of reperfusion, rats were sacrificed for the gene and protein expression NF-κB p65, COX-2, and iNOS. Also, neurological deficits and infarct volume were measured. The Ethic approval Code is IR.KMU.REC.1401.078.

Establishment of cerebral ischemia-reperfusion
Focal cerebral ischemia model was measured as described previously [26]. In brief, anesthesia was administered with ketamine–xylazine (90 mg/kg; 10 mg/kg). Lubricant Eye Drops were used to prevent dryness during surgery. After shaving, we performed a midline incision in the neck. We exposed the left common carotid artery (CCA), the external cerebral artery (ECA) and the internal cerebral artery (ICA). After occlusion of right MCA for 60 minutes, the nylon suture was removed, and cerebral blood flow was restored.

**Evaluation of neurological function**

Neurological severity scores (NSS) were performed as described in previous studies for functional sensory, motor, reflex, and balance appraisal [27]. The NSS was used to appraise sensorimotor dysfunction by grading the score on a scale of 0–18. Score descriptions are presented in Table 1.

(Table 1)

**Evaluation of infarct size**

The rats were sacrificed to assess stroke volume, and their brains were frozen for 20 min at -20°C. Then, brain matrix was used to cut the brain into 1-mm-thick coronal samples and put in 2% TTC at 37°C in a water bath for 15 min and fixed with 4% paraformaldehyde. Brain tissue slices were photographed using a digital camera (Canon, Melville, NY). The infarct area was quantified using image analysis software (NIH Image Analyzer).

**Real-Time PCR Assay**

Total RNA was extracted using Trizol reagent (GeneAll Biotechnology, Korea). According to the manufacturer’s instructions, determine the concentration through Nanodrop spectrophotometry (Thermo Scientific, Germany) at 260 nm. The synthesized cDNA was used for the mRNA assay of iNOS, COX-2, and NF-κBp65 with RT-PCR. cDNA was amplified using Reverse Transcription Kit (Qiagen, Hilden, Germany), a three-step program. GAPDH (housekeeping gene) uses for normalizing data. The forward primer for rat iNOS was: 5′ CTGGAGTTGGAGAGGATTGTG-3′; iNOS reverse, 5′ GATAGGGCCAGGAGGAGGT-3′; COX-2 forward, 5′-CTCTTCCCTCCGCTTTGTCT-3′; COX-2 reverse, 5′- CAGAGATTGGGTTTCATTAGA-3′; NF-κBp65 forward, 5′-GGCGCCAGGACCTTACTC-3′ and reverse, 5′ TGCCGGGTCTCTACAGATT-3′ and GAPDH forward, 5′-GTGGGCGGGGATAA ATGGCAC-3′; GAPDH reverse, 5′-GGAACCCTGGGAGAGTGCTC-3′. iNOS, COX-2, and NF-κB mRNA expression were carried out using an SYBR Green and calculated using the 2−ΔΔCt method.

**Assay of NF-κB, iNOS, and COX-2 in brain tissues**

The rats were sacrificed after 24 hours of reperfusion. The brain tissues (cortex, hippocampus, and striatum) were homogenized with a homogenizer. The levels of NF-κB, iNOS, and COX-2 were assessed
with ELISA kits (MultiScience (Lianke) Biotech Co.) following the manufacture protocol.

**Immunohistochemical staining**

Immunohistochemical staining was applied on 7 μm tissue sections \(^{[13]}\). Briefly, tissue sections were incubated for 30 min at 60°C, rehydrated through a descending alcohol series, and treated with 10% hydrogen peroxide in methanol for 10 min to reduce endogenous peroxidase activity. After being washed in Tris buffer (H2NC (CH2OH)3, pH7×4), antigens were retrieved by autoclaving for 11 min in citrate buffer (C6H5Na3O7×2H2O, pH6). After washing in PBS, sections were blocked with 1% fetal bovine serum (FBS) in 0.3% TritonX-100, the following primary antibody (Abcam, UK) was used by overnight incubation at 4 °C temperature. Optimal dilution was prepared to be 1/100. Tissues were then incubated in the goat polyclonal secondary antibody (HRP) (Abcam, UK) for 30 min at room temperature with the addition of 3, 3′-diaminobenzidine (DAB, Sigma, USA) to achieve visualization of the antigen. Finally, tissue sections were counterstained with Hematoxylin (Sigma) for visualization under the microscope. Photomicrographs of sections were prepared using light microscopy at ×400 magnifications by a blinded investigator. The number of immune positive cells was counted along the transect of 400 μm length (0.160 mm \(^2\)) of CA1 area of the right hippocampus.

**Statistical Analysis**

Statistical data analysis was carried out using GraphPad Prism (version 9.0; GraphPad Software, Inc.). samples obtained from the volume of tissue damage were measured using ImageJ software. One-way analysis of variance followed by Dunn's multiple comparison test showed differences between groups. All data were expressed as mean ± SEM. P values of 0.05 were considered significant differences.

**Results**

**Effect of alpha-pinene on neurological deficits in MCAO rats**

Neurologic symptoms were assessed one day after cerebral ischemia-reperfusion. A neurological defect score was performed to determine whether α-pinene treatment improved neuronal function after ischemia. Neurological deficits were observed in the MCAO group. The results showed that the neurologic deficit scores (NDS) in the MCAO group treated with α-pinene at 50, 100 mg/Kg were significantly reduced compared to the control group (\(P < 0.01, P < 0.001\)). It, therefore, shows that α-pinene improves brain dysfunction (Figure 2A).

**Effect of alpha-pinene on infarct volume in MCAO rats**

The infarct volume of mice was assessed by TTC staining. The results showed that no lesion was observed in the sham group. In the MCAO group, 24-hour reperfusion caused infarction in the cortex, hippocampus, and striatum (Figure 2B). In contrast, α-pinene at doses of 50 and 100 mg/kg significantly
reduced infarct volume compared with the control group \((P< 0.01, P < 0.001)\) (Figure 2C and 2D, 2E). These results show that α-pinene reduces damage in ischemic areas.

**Effect of alpha-pinene on the mRNA expression levels of iNOS, COX2 and NF-KB in MCAO rats**

We measured the mRNA expressions of NF-KB, iNOS, and COX2 in the mentioned brain areas using qPCR. The results of this study showed that cerebral ischemia significantly increased the expression levels of iNOS and COX-2, NF-KB in the cortex, hippocampus, and striatum compared to the sham group. Alpha-pinene significantly ameliorated iNOS, COX2 and NF-KB mRNA levels in three areas of the rat brain compared with the control group \((P <0.01, P <0.001)\) (Figure 3A, 3B and 3C).

**Effect of alpha-pinene on the protein expression levels of iNOS, COX2 and NF-KB in MCAO rats**

The effects of alpha-pinene on iNOS, COX2 and NF-KB protein expression in all five groups were investigated using ELISA. The increase in iNOS, COX2 and NF-KB levels of MCAO in mice decreased significantly after administration of alpha-pinene (100 mg/kg) \((P <0.01, P <0.001)\) Figure 4A, 4B and 4C). The present study showed that alpha-pinene (100 mg/kg) had the best protective effects in ischemic rats.

**Effect of alpha-pinene on the protein expression level of caspase-3 in MCAO rats**

The results of caspase-3 immunohistochemical staining indicated that, there is a significant difference among groups with respect to the number of active caspase-3 cells in the CA1 area of the rats’ right hippocampus. The number of active caspase-3 cells was significantly increased in ischemic group compared to sham group \((P < 0.01)\). In the alpha-pinene (100 mg/kg) treatment group, the number of active caspase-3 cells in CA1 area was significantly lower than that seen in ischemic rats \((P<0.01)\) (Figure 5, Figure 6).

**Discussion**

cerebral ischemia-reperfusion is one of the main causes of mortality and neurofunction deficits worldwide. In the current study, alpha-pinene significantly alleviates infarct volume and neurofunction deficits in cerebral ischemia/reperfusion injured rats. Its neuroprotection effect may be associated with the inhibition of inflammation or apoptosis. In this study, our results show that alpha-pinene can improve neurological dysfunction, decrease infarction area and neuronal inflammation, and decrease the level of caspase-3.

Cerebral ischemia/reperfusion injury is often accompanied with inflammation. An exacerbation of inflammatory response often leads to the activation of toxic enzymes and activation of the apoptotic cascade. Inflammation after ischemic stroke has been important rolled in the pathogenesis of brain ischemia and deteriorates brain damage. Neuroinflammatory response accompanied with the production of reactive oxygen species (ROS), cytokines and chemokines. NF-κB is an inducible transcription factor expressed in neurons, glial cells, and endothelial cells \[^{28}\]. Several researches have shown that NF-κB
plays a critical role in the inflammation after cerebral ischemia [29]. Thus, repressing NF-κB decreased cerebral ischemia [30]. Several studies have indicated that cerebral ischemia results in activation of NF-κB in neurons of the cortex and striatum [31]. Evidence has shown that NF-κB is activated in CA1 hippocampal neurons following global and focal ischemia in rats [29]. NF-κB was known to regulate inflammatory mediators such as TNF-α, IL-1β, and iNOS. NF-κB is the main actor in post-inflammation processes [32]. The findings has shown that at the transcriptional level, caspase-3 expression is regulated by NF-κB activation. Inhibition of NF-κB could be reduced the expression of many pro-inflammatory genes. Previous studies showed that alpha-pinene decreased the expressions of inflammatory factors, for instance TNF-α, IL-1β, and IL-6 [33]. Consistent with these reports, our study presented that the induction of cerebral ischemia-reperfusion caused a remarkable elevation of gene and protein levels of inflammatory factors, including NFκB p65, iNOS, and COX-2 in the brain. Alpha-pinene reduced the activities of NF-κB p65 unit, iNOS, and COX-2 mRNA and protein expression in the cortex, hippocampus, and striatum in the cerebral ischemia-reperfusion injured model rats effectively, suggesting the persistent suppression of inflammatory factors. A study has shown that alpha-pinene has anti-inflammatory effects by inhibiting inflammatory mediators such as IL-1β, NF-κB, and LTB4 [22-34]. Other findings indicated that alpha-pinene reduced the expression levels of pro-inflammatory cytokines in cerebral ischemia [35]. As well, Receptor-interacting-serine/threonine-protein kinase 2 (RIPK2) is a potent activator of NF-κB and inducer of apoptosis which α-pinene inhibits activity [36].

The hippocampus, particularly the pyramidal cell of CA1 area, is absolutely vulnerable to ischemic insults. At the time of cerebral ischemia, in addition to neuronal loss in the hippocampus, the apoptosis process is observed. It appears that the hippocampus has an important role for memory and learning [13]. Thus, the memory deficits that were happening frequently after cerebral ischemia are often related to the impairment of hippocampus function. In the current study, we demonstrated for the first time that alpha-pinene, significantly reduced caspase-3 activation in the CA1 cells after cerebral ischemia.

In the early stages of cerebral ischemia, leukocytes (neutrophils, monocytes, and lymphocytes) are expressed on endothelial cells and invade the damaged parenchyma [37]. Therefore, the accumulation of leukocytes leads to further injury. There is considerable evidence for the harmful role of iNOS and COX2 in neutrophils in cerebral ischemia. In one study, alpha-pinene showed neuroprotective and anti-inflammatory effects by reducing interleukin-1 beta and TNF-α in the cortex and striatum. Research has shown that induction of iNOS occurs after cerebral ischemia, and NO could play a pivotal role in cell injury after cerebral ischemia [38].

Research has shown that iNOS expression increases after cerebral ischemia, so inhibition of iNOS may reduce the severity of the cerebral ischemic injury [39]. Our previous findings showed that alpha-pinene neuroprotective activity may be due to its attenuating effect on NO [24]. The present research demonstrated that iNOS mRNA levels was increased during cerebral ischemia, and treatment with alpha-pinene decreased iNOS mRNA in ischemic brain tissue of rats after cerebral infarction. This observation is consistent with Kim D-S et al. [22], who demonstrated that alpha-pinene inhibited iNOS expression in
LPS-stimulated macrophages. Therefore, alpha-pinene significantly decreased iNOS mRNA and protein expression. COX-2 is a key enzyme that involves prostaglandin synthesis. COX-2 expression has also been illustrated after cerebral stroke, and inhibition of COX-2 reduces brain damage in experimental models. In the present research, the COX-2 expression has been demonstrated to increase the mRNA and the protein levels in the cortex, hippocampus and striatum after brain ischemic but reduced by α-pinene treatment.

Apoptosis is a key mechanism of cell death, which is characterized by chromatin condensation and cell division into apoptotic bodies. The proportion between Bcl-2 and Bax determined the destiny of the cells to apoptosis or not. Under the condition of cerebral ischemia, Bax is activated the apoptotic pathway, triggering the caspase-3 cascade, ultimately leading to DNA degradation and brain injury. Previous studies indicated that alpha-pinene decreased the expressions of proapoptotic factor (Bax). The inhibition of cell apoptosis can alleviate cerebral ischemia-reperfusion injury and thereby have a protective effect on brain tissues. Our findings are compatible with these results. The present study showed that alpha-pinene protected cerebral ischemia-reperfusion injury by inhibiting the activation of caspase 3 in the CA1 hippocamp area thus preventing apoptosis in the ischemia-reperfusion-injured model rats.

The findings revealed that inflammation (NF-κB, iNOS and COX-2) and apoptosis (caspase-3)-related markers were significantly reduced in the alpha-pinene-treated MCAO group compared with the control group.

Conclusively, our results demonstrated that α-pinene shows a neuroprotective effect through the regulation of iNOS -NF-kappa B- COX-2 inflammation mediators response after ischemia. Also, alpha-pinene reduces neuronal injuries of hippocampal CA1 neurons after cerebral ischemia through the reduction of caspase-3 activation. The potential mechanisms of α-pinene toward the neuroprotective may be the anti-oxidative, anti-inflammation, and anti-apoptotic effects in ischemia-reperfusion injured rats. Since alpha-pinene may influence cerebral ischemia through various procedures, much more research on other inflammatory or apoptotic markers is warranted.

**Declarations**

**Acknowledgement**

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**Author contributions**

M.K. conceived of the presented idea. M.K. and S.E. designed and directed the project. M.K. and F.E. performed the experiments. All authors discussed the results and contributed to the final manuscript.

**Declarations**
Conflict of interest

All authors of this manuscript say that they have no conflicts of interest to disclose.

Ethical approval

All laboratory animals were carried out with specific consideration of EU directive 2010/63/EU. The Ethic approval Code is IR.KMU.REC.1401.078.

References


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Tables

Table 1
<table>
<thead>
<tr>
<th>Behavior Tests</th>
<th>Scores</th>
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<tbody>
<tr>
<td>Raising the rat by the tail</td>
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<tr>
<td>Head moved more than 10° to the vertical axis within 30 s</td>
<td>1</td>
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<tr>
<td>Flexion of forelimb</td>
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<tr>
<td>Flexion of hindlimb</td>
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<td>Sensory tests</td>
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<td>The tactile and visual placing test</td>
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<tr>
<td>The proprioceptive senses (deep sensation, pushing the toe against the table edge to stimulate limb muscles)</td>
<td>2</td>
</tr>
<tr>
<td>Movement tests</td>
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<tr>
<td>Normal walk</td>
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<tr>
<td>Inability to walk straight</td>
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</tr>
<tr>
<td>Circling toward the paretic side</td>
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</tr>
<tr>
<td>Fall down to one side</td>
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<tr>
<td>staring and Immobility</td>
<td>1</td>
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<td>Beam balance tests</td>
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<tr>
<td>Balances with steady posture</td>
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<tr>
<td>Grasps side of the beam</td>
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</tr>
<tr>
<td>Hugs the rod and falling down one limb from the rod</td>
<td>2</td>
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<td>Hugs the rod and falling down two limbs or spins on beams (&gt;60 s)</td>
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<tr>
<td>Attempts to balance on the rod but falls off (&gt;40 s)</td>
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<tr>
<td>Attempts to balance on the rod but falls (&gt;20 s)</td>
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<tr>
<td>Falls off: without any attempts to balance or hang on to the rod (&lt;20 s)</td>
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<td>Reflex Activity</td>
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<td>Corneal reflex (an eye blink when touching the cornea with cotton)</td>
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<td>Pinna reflex (shaking the head when touching the auditory duct)</td>
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<td>Startle reflex (motor response to a brief noise from snapping a clipboard paper)</td>
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<td>Myodystony, irritability, Myoclonus and Seizures</td>
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**Figures**
Figure 1

Chemical structure of alpha-pinene.
**Figure 2**

**Effect of alpha-pinene on neurobehavioral function (A), the size of cerebral infarct (B and C), following cerebral ischemic stroke in rats.** Control (normal saline + tween 80) and α-pinene were administered at the beginning of reperfusion. (A) The neurological score of MCAO groups and α-pinene groups. (B) Representative photographs showing the cerebral infarct of rat coronal brain sections measured by (TTC) staining. The regions with white color indicate ischemic portions. (C, D and E) The infarct size of MCAO groups.
groups and α-pinene groups. All data were expressed as mean±SEM. **P < 0.01, ***P < 0.001 (compared to MCAO group); ###P < 0.001 (compared to sham group).

Figure 3

Alpha-pinene inhibits the mRNA expression of COX-2, iNOS and NF-κB, in the MCAO model. Alpha-pinene (100 mg/kg) was administered i.p. after MCAO at the beginning of reperfusion. (A, B and C) The mRNA expression of COX-2, iNOS and NF-κB was determined in the cortex, hippocampus, and striatum by 24 h
after reperfusion. Compared to the MCAO group, the alpha-pinene treatment also decreased the mRNA expression of COX-2, iNOS and NF-κB. All data were expressed as mean±SEM. *P < 0.05, **P < 0.01, ***P < 0.001, vs MCAO; ##P < 0.01, ###P < 0.001, vs Sham.

Figure 4

Alpha-pinene inhibits the protein expression of COX-2, iNOS and NF-κB in the MCAO model. Alpha-pinene (100 mg/kg) was administered i.p. after MCAO. (A, B and C) The ELISA method measured the protein expression of COX-2, iNOS and NF-κB in the cortex, hippocampus and striatum. Compared to the MCAO
group, the alpha-pinene treatment also decreased the protein levels of COX-2, iNOS and NF-κB. All data were expressed as mean±SEM. *P < 0.05, **P < 0.01, ***P < 0.001, vs MCAO; ##P < 0.01, ###P < 0.001, vs Sham.

Figure 5

Alpha-pinene inhibits the protein expression of Caspase-3 in the MCAO model. The immunohistochemical staining measured the protein expression of Caspase-3 in the CA1 area of the right hippocampus. (A) The CA1 area of hippocampus, (B) sham group, (C) MCAO group, (D) Alpha-pinene (100 mg/kg) group (Arrows indicate the active caspase-3 cells, Magnification x400). All data were expressed as mean±SEM. *P < 0.05, **P < 0.01, ***P < 0.001, vs MCAO; ##P < 0.01, ###P < 0.001, vs Sham.
Figure 6

Effects of Alpha-pinene on the number of active caspase-3 cells in the rat's hippocampal CA1 area in the MCAO model. Alpha-pinene significantly attenuated the ischemia/reperfusion-induced caspase-3 activation. All data were expressed as mean±SEM. *P < 0.05, **P < 0.01, ***P < 0.001, vs MCAO; ##P < 0.01, ###P < 0.001, vs Sham.

Supplementary Files

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