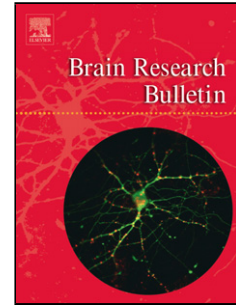


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Neuroprotective effect of morroniside on focal cerebral ischemia in rats

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ABSTRACT

Cornus officinalis Sieb. et Zucc., known as Shan-zhu-yu in Chinese, has been used to treat cerebrovascular disease and diabetes in Traditional Chinese Medicine for a long time and morroniside is the main component of Shan-zhu-yu. In this study, we examined whether morroniside could protect ischemia/reperfusion-induced brain injury by minimizing oxidative stress and anti-apoptosis. Morroniside was intragastrically administered to rats in doses of 30, 90 and 270 mg/kg/day, starting 3 h after the onset of middle cerebral artery occlusion. The behavioral test was performed by using the Zea-Longa scores, Prehensile Traction score and Ludmila Belayer score. Rats were sacrificed 3 days after ischemia occurred. The infarction volume of brain was assessed in the brain slices stained with 2, 3,

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5-triphenyl tetrazolium chloride. Cortex tissues were also used for determination of malondialdehyde levels, glutathione levels and superoxide dismutase. The treatment with morroniside significantly improved Zea-Longa scores and Prehensile Traction score at the doses of 30, 90 and 270 mg/kg, increased Ludmila Belayev score and reduced the infarction volume at the doses of 90 and 270 mg/kg. Morroniside (30, 90 and 270 mg/kg) treatment significantly decreased the level of malondialdehyde and caspase-3 activity by colorimetric analysis in ischemic cortex tissues. Morroniside (270 mg/kg) treatment significantly increased the content of glutathione, enhanced the activity of superoxide dismutase, but decreased the caspase-3 expression by Western-blot analysis in ischemic cortex tissues. These findings demonstrated that morroniside could notably protect the brain from damage induced by focal cerebral ischemia which might be related to morroniside antioxidant and anti-apoptotic properties in the brain.

Keywords: Anti-apoptotic; Antioxidant; Cerebral ischemia; Middle cerebral artery occlusion;

Morroniside; Neuroprotection

1. Introduction

Cerebral ischemia is caused by a reduction of the cerebral blood flow leading to a cascade of pathological mechanisms such as loss of ionic homeostasis, energy failure, excessive release of excitatory amino acids, increased oxidative stress, apoptosis and finally irreversible tissue damage [11]. Oxidative stress is one of the primary factors that exacerbate damage caused by cerebral ischemia [5]. Superoxide and hydroxyl radical are potent in producing destruction of the cell membrane by inducing lipid peroxidation. The brain is particularly vulnerable to oxidative stress injury because of its high rate of oxidative metabolic activity and intense production of reactive oxygen species metabolites, and its high content of polyunsaturated fatty acids, relatively low antioxidant capacity [9].

Neuroprotective agents acting on individual neurotoxic mechanisms have been tested with promising success in animal models, but have failed to benefit patients in clinical trials. Traditional Chinese medicines have been used to treat stroke for years, and there have been some reports about the mechanisms underlying their neuroprotective actions [10, 12, 15, 25, 33, 35]. *Cornus officinalis* (Shan-zhu-yu), considered one of the 25 plant-based drugs most frequently used in China, Japan, and Korea [26], has been highly prized for many years for its ability to treat cerebrovascular and cardiovascular diseases. The total iridoid glycoside content of *C. officinalis*, including loganin, morroniside and other minor constituents, possesses a number of pharmacological activities such as mitigating the vascular

complications of diabetes, depressing immunity, rheumatoid arthritis and cerebral ischemia-reperfusion injury through multiple mechanisms of action, against diabetic renal damage, regulator of hepatic inflammatory reactions and lipid metabolism et al. [13, 16, 21, 23]. Morroniside is one of the most abundant iridoid glycosides in *C. officinalis* and its chemical structure is shown in Fig. 1. We have previously reported that incubation of SH-SY5Y cells with morroniside leads to a significant dose-dependent elevation of cellular glutathione accompanied by a marked protection against H₂O₂-mediated toxicity, inhibits the formation of reactive oxygen species, depress the activation of caspase-3 and -9 but upregulates Bcl-2 [29, 30].

Therefore, in the present study, we have evaluated whether morroniside is effective in producing neuroprotection, as assessed by neurobehavioral tests, infarct size measurement, antioxidant and anti-apoptotic properties following 30 min of middle cerebral artery occlusion (MCAO) in adult rats.

2. Materials and methods

2.1. Morroniside Extraction

The sarcocarp of *C. officinalis* was purchased from Tong-Ren-Tang Company, Beijing, China, and authenticated by Professor Wen Wang. The air-dried material was powdered and extracted with water, and the resulting solution concentrated to produce an aqueous residue. The residue was extracted with 80% ethanol, and the resulting solution was again extracted

with ethanol. After dissolving the residue in water, the aqueous solution was concentrated and chromatographed on an SP70 resin column using 20% EtOH as the eluant. The 20% EtOH eluate was concentrated and then chromatographed on a silica gel column using CHCl₃-MeOH eluant mixtures (15:1-8:1) to yield a crude iridoid glycoside fraction. This fraction was re-chromatographed on a silica gel column using CHCl₃-MeOH eluant mixtures (10:1-6:1) to generate a white crystalline product. High performance liquid chromatography (HPLC) analysis consisted of a YMC Co. (Dinslaken, Germany) C₁₈ column (250 mm × 4.6 mm, 10 μm) at a column temperature of 35 °C. Glycosides were eluted with an acetonitrile-water mixture (15:85) at a flow rate of 1.0 ml/min and a detection wavelength of 240 nm. Their final purity was determined to be 98.5%.

2.2. *Animals and middle cerebral artery occlusion*

Male Wistar rats weighing from 250 to 270 g were purchased from Beijing Vitalriver Experimental Animal Co., Beijing, China, and were housed under a 12/12 h dark/light cycle and specific pathogen-free (SPF) conditions. During the entire experiment, the rats had free access to food and water. They were randomly divided into five groups (n=14 in each group): (1) a sham-operated group, (2) a vehicle-treated ischemic model group, (3) a 30 mg/kg/day morroniside-treated ischemic group, (4) a 90mg/kg/day morroniside-treated ischemic group, and (5) a 270 mg/kg/day morroniside-treated ischemic group.

The middle cerebral artery occlusion was induced by the intraluminal filament technique [18]. Rats were anesthetized with 10% chloral hydrate (0.4 ml/kg, IP). Then, a piece of nylon monofilament was inserted into the left internal carotid artery. After 30min of ischemia, the filament was withdrawn. In sham-operated animals, the middle cerebral artery (MCA) was not occluded. All experimental procedures were carried out in accordance with the Provisions and General Recommendations of Chinese Experimental Animal Administration Legislation, and all efforts were made to minimize both the number of animals used and any suffering by the animals.

Morroniside was dissolved in normal saline and administered intragastrically once a day at the doses of 30, 90, and 270mg/kg, respectively, starting 3 h after MCAO. The vehicle control groups of ischemic rats and sham-operated rats received an equal volume of normal saline.

2.3. *Neurobehavioral functional scoring*

The neurological scoring systems proposed by Li and Belayer [2, 14] were adopted with modifications, as shown in Table 1 and Table 2. All neurological evaluations were done by a researcher blinded to knowledge of the animal groupings. Neurological assessment was composed of three test scores: Zea-Longa score, Prehensile Traction test score, and Ludmila Belayer score. A higher score in each test represents more severe deficits.

(Table 1, Table 2)

2.4. *Measurement of infarct volume*

Infarct volume was assessed using 2, 3, 5-triphenyltetrazolium chloride (TTC) (Sigma, St. Louis, MO) staining. 3 days after reperfusion, rats were injected with 120 mg of pentobarbital. The brain was then removed and cut into 2 mm sections. The slices were placed in a petri dish containing 0.05% TTC for 30 min at 37°C, and periodically agitated to ensure that no slices were resting on the bottom, and then put into 10% formaldehyde. Lesion volumes were calculated from summed, measured areas (Sigma Scan Pro, SPSS software) of unstained tissue in mm² multiplied by the 2-mm slice thickness. The individual measuring the infarct size was blinded as to whether vehicle or morroniside had been administered.

2.5. *Measurement of superoxide dismutase(SOD) activities, glutathione (GSH) and malondialdehyde (MDA) levels in brain microvascular homogenates*

After 3 days of reperfusion, rat brain capillary fragments were isolated using modified methods introduced by Abbott and Lin [1, 17]. Briefly, fresh brain hemispheres were dropped into ice-cold Buffer A (10 mM HEPES, 11.9 mM NaHCO₃, 140 mM NaCl, 10 mM KCl, 0.1% BSA), and the cerebellum, brain stem, choroid plexus, and meninges were carefully removed. The cortices were chopped with a scalpel for <1 min into uniform 2-3 mm pieces in Buffer A, digested in a dispase solution (Ca²⁺, Mg²⁺-free HBSS with 20 units/ml DNase I) for 1 h at 37 °C to separate microvessels from other components, and the solution was then centrifuged in 15% dextran at 4500×g, at 4 °C for 20 min. The pellet containing crude

microvessels was resuspended in PBS and was diluted to 1mg protein/ml. Protein content was determined with BSA as a standard according to Bradford [4] and the homogenates were used immediately for assays of malondialdehyde (MDA), glutathione (GSH) and superoxide dismutase (SOD).

Malondialdehyde, glutathione levels and superoxide dismutase activities were determined following the kit instructions (Jiancheng Institute of Biotechnology). Malondialdehyde was determined by the thiobarbituric acid method [22]. The assay for total superoxide dismutase was based on its ability to inhibit the oxidation of oxyamine by the xanthine-xanthine oxidase system. Tissue levels of glutathione were assayed colorimetrically at 412 nm [8].

2.6. *Caspase-3 Activity Assay*

Activity of caspase-3 was detected with a commercially available caspase-3 activity kit (Beyotime Institute of Biotechnology), with Ac-DEVD-pNA as the colorimetrically specific substrate. In brief, right cortical samples (n=6 for each group) were weighed and homogenized in lysis buffer containing 10 mM/L HEPES/KOH (pH7.2), 2 mM/L EDTA, 0.1% CHAPS, 5 mM/L dithiothreitol, 1 mM/L phenylmethylsulfonylfluoride, 10 µg/mL aprotinin, and 20 µg/mL leupeptin. The lysate was centrifuged at 20 000g for 10 minutes at 4°C, and supernatants were incubated for 7 hour at 37°C with 10 µL caspase-3 substrate (Ac-DEVDpNA, 2 mM/L). Substrate cleavage was measured with a spectrofluorometer at

405 nm and was corrected as protein content in the lysate. The activity of caspase-3 was expressed as values of enzyme activity compared with control [31].

2.7. Western-blot analysis

After day 3 of reperfusion, each rat was sacrificed under 10% chloral hydrate (0.4 ml/kg) anesthesia. The infarct side of the cortex (1-5 mm posterior to the bregma and 1-5 mm lateral to the sagittal suture) was harvested for the assay of protein expression of activated caspase-3. The brain tissue was homogenized in an ice-cold buffer (tris-(hydroxymethyl)-aminomethane 50 mM, pH 7.4, NaCl 150 mM, 0.5% TritonX-100, edetic acid 1 mM, phenylmethylsulfonyl fluoride 1 M, and aprotinin 5 mg/L), centrifuged at 14,000×g at 4 °C for 30 min and the supernatants was then collected as total protein. Proteins were electrophoresed through a 15% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), and electrically transferred to a nitrocellulose membrane. This membrane was incubated at 4 °C overnight in tris- (hydroxymethyl)- aminomethane buffered saline (TBS) containing 5% milk, and detected with the primary rabbit polyclonal antibody against cleaved caspase-3 of rat (at 1:1000 dilution, Santa Cruz Biotechnology, U.S.A.). After washing with TBS, membranes were incubated with secondary antibody (horseradish peroxidase conjugated anti-rabbit IgG) at room temperature for 1 h. Gels were scanned and quantified, and the amount was normalized with β -actin values in the same lane.

2.7. Statistical analysis

Data were presented as means \pm SEM. The statistical significance between means was calculated by analysis of variance (ANOVA) followed by Tukey's multiple range post-hoc test. "*P*" values less than 0.05 were assumed to denote a significant difference using SPSS 13.0 Statistical Software.

3. Results

3.1. Effect of morroniside on neurological function after ischemia-reperfusion

Infarction of the cerebral cortex caused a deficit in neurological function in the rats that was mainly visible as left forelimb paralysis. Behavioral deficits were evaluated by means of three neurological function tests: the Zea-Longa score, the Prehensile Traction test and the Ludmila Belayev score. A higher score in each test represents a more severe deficit. The Zea-Longa scores in the vehicle-treated group was significantly higher than that in the sham-operated rat from 0 to 3.0 ± 0.52 ($P < 0.001$, $n=6$), but the Zea-Longa scores was obviously depressed from 3.0 ± 0.52 to 1.7 ± 0.21 ($P < 0.05$, $n=6$), 1.5 ± 0.22 ($P < 0.01$, $n=6$), and 1.3 ± 0.33 ($P < 0.01$, $n=6$) respectively when treatment with morroniside different concentration (30, 90, 270mg/kg/day) at 3 days after MCAO (see Fig. 2). As we see, Prehensile Traction test of rats in the vehicle-treated group were significantly higher than that in the sham-operated rat from 0 to 1.2 ± 0.48 ($P < 0.001$, $n=6$), however, the Prehensile Traction test of rats in the morroniside-treated group (30, 90, 270mg/kg/day), but morroniside-treated group at 10mg/kg/day, were significantly depressed to 0.2 ± 0.17 ($P <$

0.01, n=6) at 3 days after MCAO (Fig. 3). Ludmila Belay scores of the vehicle-treated group was manifestive increased to 7.8 ± 0.75 ($P < 0.001$, n=6), however, when treatment with morroniside (90, 270mg/kg), Ludmila Belay scores was notable decreased to 5.0 ± 0.73 ($P < 0.05$, n=6), 3.8 ± 0.98 ($P < 0.01$, n=6) respectively. These results suggested that it had considerable improvement in neurological function at 3 days after MCAO compared to that seen in the vehicle-treated group, an indication that the neurological function of MCAO rats can be restored by treatment with morroniside (90, 270mg/kg). No neurological function deficits were seen in the sham-operated group (Fig. 4).

3.2. Effect of morroniside on infarct volume after ischemia-reperfusion

In order to further examine the neuroprotective effect of morroniside against an ischemia/reperfusion insult, we measured the infarct volume with or without administration of morroniside. As shown in Fig. 5, infarct volume was 26.88 ± 0.03 % in vehicle-treated animals, it was significantly reduced by 16.07 % to 22.56 ± 0.03 % ($P < 0.05$, n=6) and 61.79 % to 10.27 ± 0.02 % ($P < 0.001$, n=6) respectively, in animals given a 90 , 270mg/kg dose of morroniside. These observations indicate that morroniside can degrade ischemia/reperfusion induced brain injury.

3.3 Effect of morroniside on the content of malondialdehyde after ischemia-reperfusion

3 days after 30 min of forebrain ischemia, malondialdehyde content, an index of lipid peroxidation, was significantly elevated in ischemia-subjected rats compared to that in

sham-operated group from 10.0 ± 0.85 to 14.7 ± 0.97 nmol/mg ($P<0.01$, $n=6$).

Ischemia-mediated lipid peroxidation was significantly decreased to 11.2 ± 0.93 nmol/mg ($P<0.05$, $n=6$), 10.7 ± 1.31 nmol/mg ($P<0.01$, $n=6$), 9.0 ± 0.97 nmol/mg ($P<0.001$, $n=6$), respectively in morroniside-administered (30, 90, 270 mg/kg) rats compared to the ischemia-subjected group, as shown in Fig. 6.

3.4. Effect of morroniside on glutathione content after ischemia-reperfusion

3 days after 30 min of forebrain ischemia, cortex glutathione levels decreased by 36.36% from 0.022 ± 0.003 to 0.014 ± 0.002 mg/g ($P<0.05$, $n=6$) compared to those seen in the sham-operated groups. However, when treatment with morroniside (270 mg/kg) significantly enhanced glutathione levels about 33.33% to 0.021 ± 0.003 mg/g ($P<0.05$, $n=6$) compared to the the vehicle-treated ischemic rats (see Fig. 7).

3.5. Effect of morroniside on activities of superoxide dismutase after ischemia-reperfusion

The results of superoxide dismutase activities are summarized in Fig. 8. The activity of superoxide dismutase in the cortex was decreased by 6.67% from 126.3 ± 5.58 to 118.4 ± 13.77 U/mg ($P<0.05$, $n=6$) in ischemic rats compared to sham-operated rats. However, when treatment with morroniside (270 mg/kg), superoxide activities was significantly increased by 29.36% to 178.8 ± 14.65 U/mg ($P<0.01$, $n=6$) compared to the vehicle-treated ischemic rats.

3.6. Effect of morroniside on caspase-3 expression and activity

As shown in Fig. 9, 3 days after ischemia-reperfusion, western blot analysis showed that the expression caspase-3 increased remarkably by 64.39% in the vehicle group from 100.00 ± 0.00 to $280.81 \pm 63.41\%$ ($P < 0.01$, $n=6$). However, expression caspase-3 was reduced in the same brain areas by morroniside treatment (270 mg/kg) by 43.86% to 157.64 ± 36.33 ($P < 0.01$, $n=6$) compared to the vehicle-treated ischemic rats.

In order to reconfirm morroniside be able to depress caspase-3 activity, colorimetric analysis was applied. As it shown in Fig. 10, caspase-3 activity in the vehicle group was significant enhanced by 510.49%, compared with the sham group. In the morroniside treatment (30, 90, 270 mg/kg) group, there was a significant decrease in caspase-3 activity by 20.49% ($P < 0.05$, $n=6$), 30.96% ($P < 0.05$, $n=6$) and 43.69% ($P < 0.001$, $n=6$) respectively, compared to that in the vehicle group.

4. Discussion

In spite of a long history of clinical application of *Cornus officinalis* (Chinese name, Shan-zhu-yu) in the treatment of ischemic disorders of the cerebrovascular disease and diabetes in Traditional Chinese Medicine, it is considered one of the 25 plant-based drugs most frequently used in China, Japan, and Korea also [26]. However, it is completely unknown which of compounds of Shan-zhu-yu are active ingredients in the treatment of cerebrovascular disease. Recent studies have shown that morroniside (chemical structure see Figure 1), a pure component from the seeds of *C. officinalis*, is valuable in the treatment of

acute ischemic stroke via multi-mechanisms, such as antioxidation, anti-apoptosis [18, 29], against diabetic renal damage, regulator of hepatic inflammatory reactions and lipid metabolism [21, 23]. Therefore, we speculated that morroniside might play a key role in the treatment of ischemic disorders of the cerebrovascular system. The data presented in this study confirmed our hypothesis.

Over the last decade, important advances have been made to support the fact that reactive oxygen species (ROS) are generated and play a harmful role during the acute and late stages of cerebral ischemia. Several drugs, such as radical scavengers and antioxidants, have been evaluated in preclinical and clinical studies. It was reported that earliest stage of acute permanent middle cerebral artery occlusion (MCAO) in rats, the level of reactive oxygen species determined by superoxide dismutase (SOD), malondialdehyde (MDA) and nitric oxide (NO) in ischemic brain tissue were separately examined at the 18, 48 and 120 hour post-ischemia time points using spectrophotometry. The results indicated that there were significant improvements in the neurobehavioral outcome of the rats in the 9 and the 18 hour groups, as compared with rats from the control group [32], just like Edaravone, a free radical scavenger, attracted increasing attention since its introduction not only because of its use in the acute stage of stroke but also because of its potential application in the treatment of other important ROS-related diseases [24, 28]. So used preventative but utilization after stroke

might be highly efficient in reducing infarct volume and improving neurobehavioral outcome in permanent MCAO rats within the earliest stages of stroke.

Treatment with morroniside can significantly reduce infarct volume. This result indicates that less histological damage is observed than that in morroniside group. Therefore, treatment with morroniside also alleviated the neurological impairment caused by MCAO.

Our results showed that there was a significant increase in the level of lipid peroxidation and decrease in superoxide dismutase activities in the brain tissues after ischemia-reperfusion. However, treatment with morroniside significantly attenuated the increased lipid peroxidation induced by FeCl_3 as well as restored the activities of superoxide dismutase in brain tissues. On the basis of the hypothesis of damage mediated by oxygen free radical, the mechanism of maintenance of glutathione homeostasis is believed to be the major form of defence of the organism against these lesions. GSH is used as a substrate for the action of the enzyme glutathione peroxidase in the removal of superoxide radicals. Our results confirm that the glutathione content was significantly reduced due to ischemic insult, this could be explained by the consumption of glutathione due to its scavenging of the rapidly generated reactive oxygen species produced in ischemia. After administered morroniside, glutathione content was obviously enhanced. Furthermore, the current work also showed that there was a significant decline in forebrain activity of the endogenous antioxidant enzymes superoxide

dismutase in ischemic rats which is conformity with previous studys [6, 7, 19]. Morroniside treatment significantly decreased the level of malondialdehyde in ischemic cortex tissues too.

Damage to neurons may occur through oxidative stress and/or mitochondrial impairment and culminate in activation of an apoptotic cascade. Apoptosis or related phenomena are possibly involved in secondary cell death in cerebral ischemia. Much remains to be done in the area of cerebral stroke to explore the potential of anti-apoptotic drugs [27]. It was therefore thought to be worthwhile to explore the effect of morroniside on apoptosis. Results from the present study indicate that morroniside exerts its major action on apoptotic activity. It is well known that caspases are a family of intracellular proteins involved in the initiation and execution of cell apoptosis. Caspases are specifically activated in response to apoptotic stimuli, and caspase-3 is believed as a final killer of apoptosis [3, 26, 34]. There is a large amount of evidence indicating that cerebral ischemia can induce the activation of caspases including caspase-3, the up-regulation , which have been found to precede neuronal death. Caspase-mediated neuronal death after transient focal cerebral ischemia is more extensive than after permanent ischemia and may contribute to the delayed loss of neurons from the penumbral region of infarcts [15, 20]. In the present study, it was found that neuron loss and increased infarction volume in the cortex of rat after transient focal brain ischemia. After administration of morroniside, expression and/or activated caspase-3 was apparently inhibited and reduced the infarction volume in rat.

In conclusion, morroniside could protect ischemia/reperfusion- induced brain injury by minimized oxidative stress, decreased the caspase-3 activity, reduced the infarction volume, and improved neurological function. The antioxidant and anti-apoptotic properties of morroniside might be contributed to its neuroprotective potential in cerebral ischemic damage.

Conflict of interest

We ensure that all authors have no competing financial interests.

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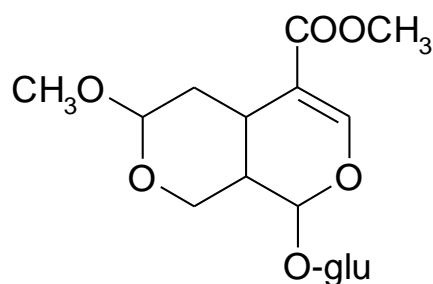


Fig. 1. Structure of morroniside.

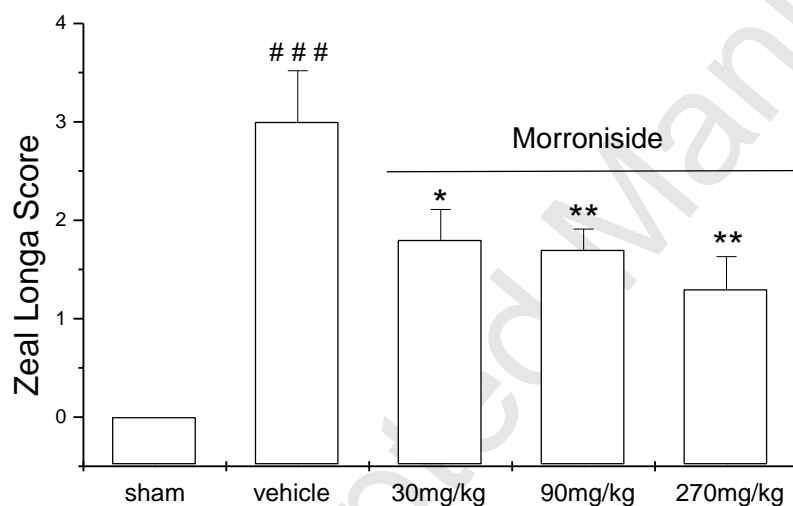


Fig. 2. Effect of morroniside on Zea Longa score in rat. Focal cerebral ischemia was induced by middle cerebral artery occlusion for 30 min followed by 3 days of reperfusion. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple range post-hoc test for multiple comparisons. Values are mean \pm SEM. Each group consists of 6 rats. ### $P < 0.001$ vs sham-operated rat; * $P < 0.05$, ** $P < 0.01$ vs vehicle-treated group.

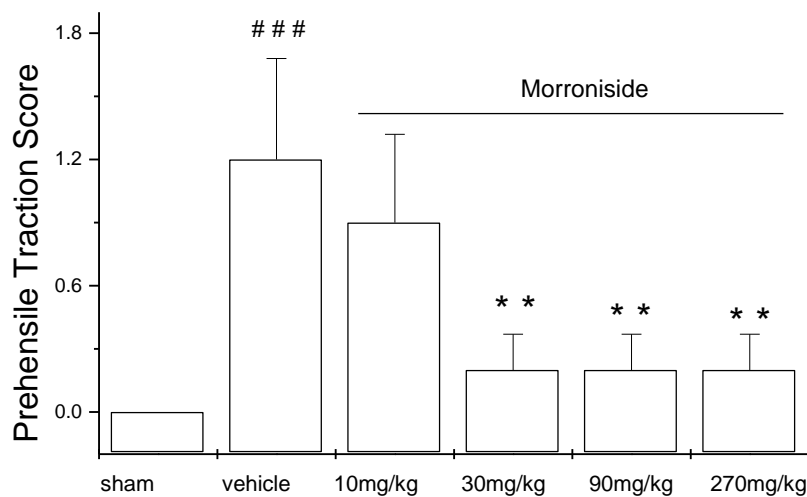


Fig. 3. Effect of morroniside on Prehensile Traction score in rat. Focal cerebral ischemia was induced by middle cerebral artery occlusion for 30 min followed by 3 days of reperfusion. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple range post-hoc test for multiple comparisons. Values are mean \pm SEM. Each group consists of 6 rats. ### $P < 0.001$ vs sham-operated rat; ** $P < 0.01$ vs vehicle-treated group.

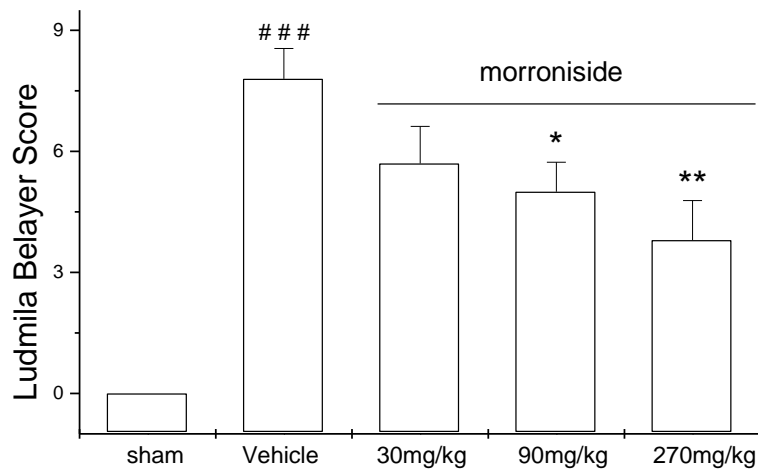
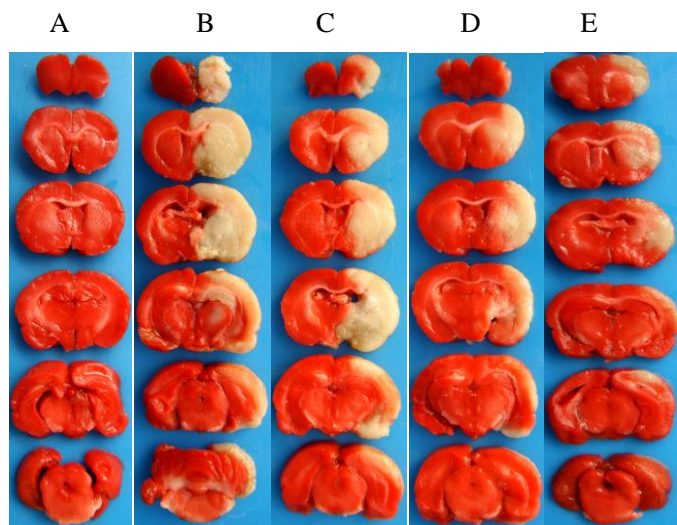


Fig. 4. Effect of morroniside on Ludmila Belayer score in rat. Focal cerebral ischemia was induced by middle cerebral artery occlusion for 30 min followed by 3 days of reperfusion. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple range post-hoc test for multiple comparisons. Values are mean \pm SEM. Each group consists of 6 rats. ### $P < 0.001$ vs sham-operated rat; * $P < 0.05$, ** $P < 0.01$ vs vehicle-treated group.



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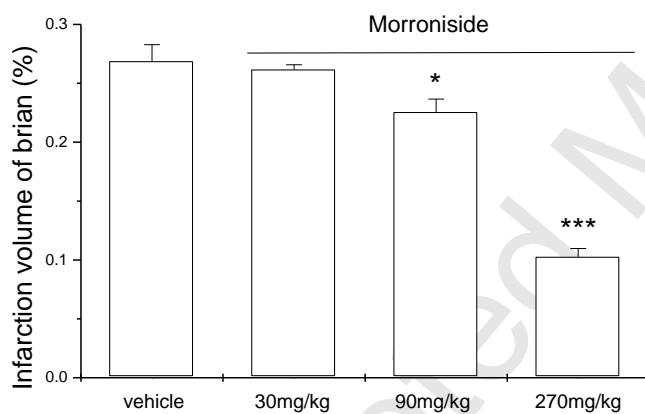


Fig. 5. Effect of morroniside on infarction volume in the brain in rat. Focal cerebral ischemia was induced by middle cerebral artery occlusion for 30 min followed by 3 days of reperfusion. (A) Sham control. (B) Vehicle groups. (C) Administration of morroniside (30 mg/kg). (D) Administration of morroniside (90 mg/kg). (E) Administration of morroniside (270 mg/kg). (F) Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple range post-hoc test for multiple comparisons. Values are mean \pm SEM. Each group consists of 6 rats. * $P < 0.05$, *** $P < 0.001$ vs vehicle-treated group.

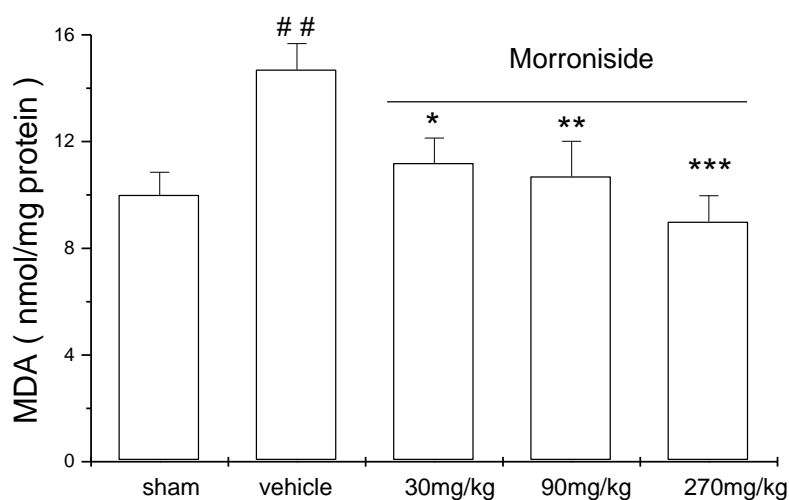


Fig. 6. Effect of morroniside on the content of malondialdehyde in rat cortex. Focal cerebral ischemia was induced by intraluminal middle cerebral artery occlusion for 30 min followed by 3 days of reperfusion. Results are expressed as mean \pm SEM of 6 rats and data were analyzed by one-way ANOVA followed by Tukey's multiple range post-hoc test for multiple comparisons. ## $P < 0.01$ vs sham-operated rat; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs vehicle-treated ischemic rat.

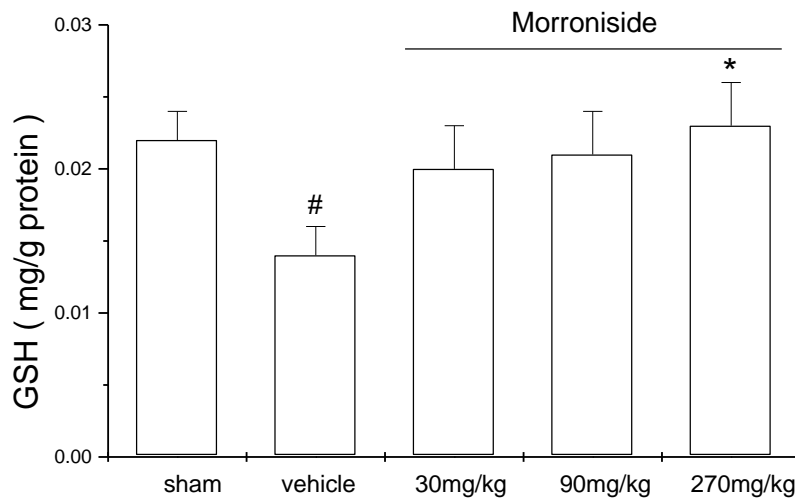


Fig. 7. Effect of morroniside on glutathione content in rat cortex. Focal cerebral ischemia was induced by intraluminal middle cerebral artery occlusion for 30 min followed by 3 days of reperfusion. Results are expressed as mean \pm SEM of 6 rats and data were analyzed by one-way ANOVA followed by Tukey's multiple range post-hoc test for multiple comparisons. # $P < 0.05$ vs sham-operated rat; * $P < 0.05$ vs vehicle-treated ischemic rat.

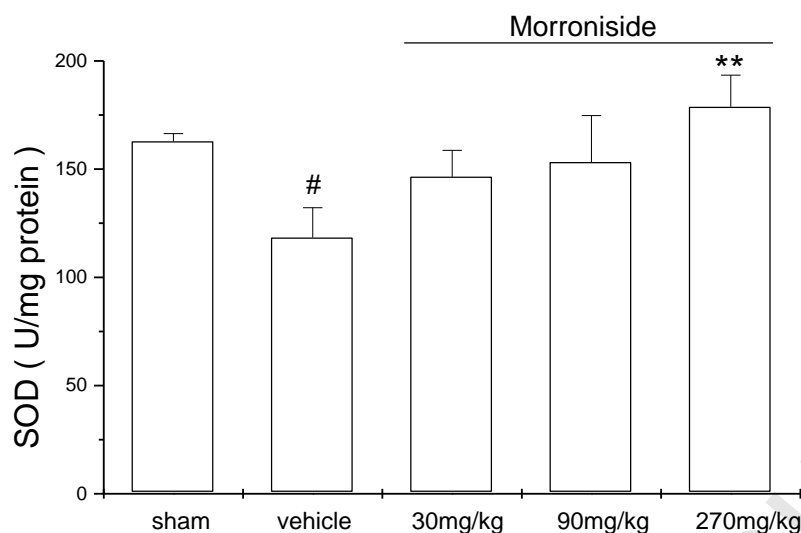


Fig. 8. Effect of morroniside on superoxide dismutase content in rat cortex. Focal cerebral ischemia was induced by intraluminal middle cerebral artery occlusion for 30 min followed by 3 days of reperfusion. Results are expressed as mean \pm SEM of 6 rats and data were analyzed by one-way ANOVA followed by Tukey's multiple range post-hoc test for multiple comparisons. # $P < 0.05$ vs sham-operated rat; ** $P < 0.01$ vs vehicle-treated ischemic rat.

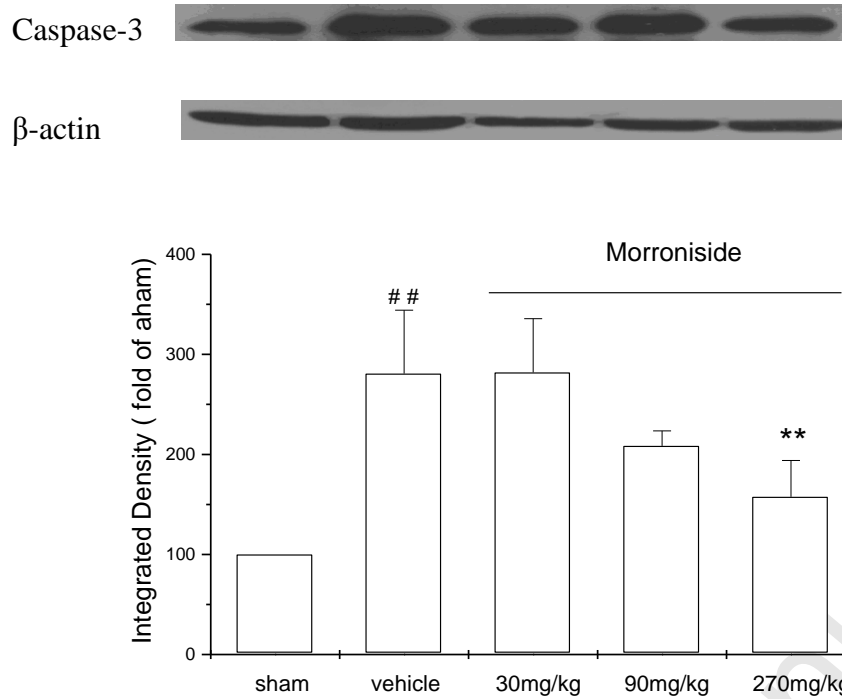


Fig. 9. Effect of morroniside on the caspase-3 expression in rat cortex. Focal cerebral ischemia was induced by intraluminal middle cerebral artery occlusion for 30 min followed by 3 days of reperfusion. Results are expressed as mean \pm SEM of 5 rats and data were analyzed by one-way ANOVA followed by Tukey's multiple range post-hoc test for multiple comparisons. ^{##} $P < 0.01$ vs sham-operated rat; ^{**} $P < 0.01$ vs vehicle-treated ischemic rat.

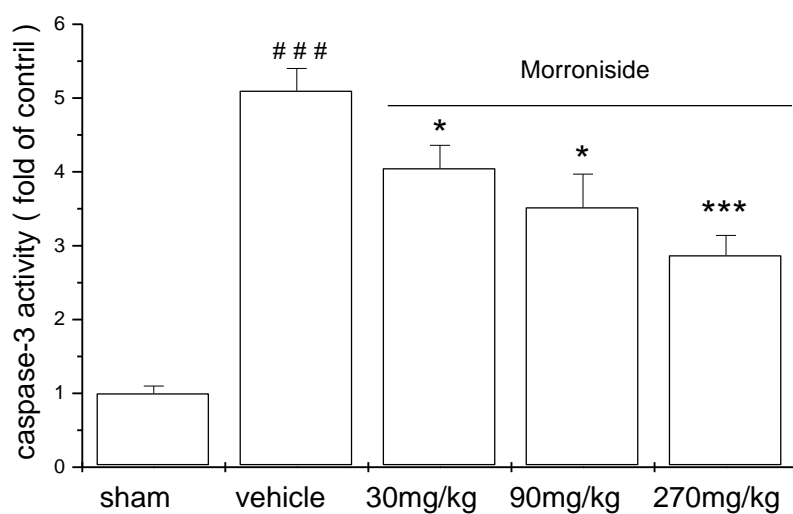


Fig. 10. Assay of morroniside on the caspase-3 activities in rat cortex. Focal cerebral ischemia was induced by intraluminal middle cerebral artery occlusion for 30 min followed by 3 days of reperfusion. Results are expressed as mean \pm SEM of 6 rats and data were analyzed by one-way ANOVA followed by Tukey's multiple range post-hoc test for multiple comparisons. $^{##}P < 0.01$ vs sham-operated rat; $^{*}P < 0.05$, $^{***}P < 0.001$ vs vehicle-treated ischemic rat.

Table 1

Neurological deficit scoring system for rats in the Zea-Longa and Prehensile Traction tests.

Zea-Longa score	
0	The rat has no neurological defect
1	The rat is unable to extend the affected fore limb
2	The rat circles while walking
3	The rat tumbles to its side while walking because of hemiplegia
4	The rat is unconscious and unable to walk
5	The rat is dead
Prehensile Traction test	
0	The rat hangs on for 5 seconds and brings rear limb up to the rope
1	The rat hangs on for 5 seconds, no rear limb brought up to the rope
2	The rat hangs on 3 to 4 seconds
3	The rat hangs on 0 to 2 seconds

Table 2

Neurological deficit scoring for rats in the Ludmila Belayev test.

Item	Normal Score	Deficit
Postural reflex ("hang test")*	0	2
Placing test (performed on each side)#		
Visual placing		
Forward	0	2
Sideways	0	2
Tactile placing		
Dorsal surface of paw	0	2
Lateral surface of paw	0	2
Proprioceptive placing	0	2
Total score	0	2

*Scores are as follows: 0, no observable deficit; 1, limb flexion during hang test; 2, deficit on lateral push.

#Scores are as follows: 0, complete immediate placing; 1, incomplete and/or delayed placing (<2 s); 2, absence of placing.

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