Benzoinum exerts NVU protective effect by inhibiting cell apoptosis in cerebral ischemia rats

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Research

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Abstract

Background

Benzoinum (Styraceae) is a traditional Chinese medicine known to treat stroke and other cardio-cerebrovascular diseases for thousands of years. Benzoinum also proved to have diverse pharmacological activity, but the neuroprotection mechanism about apoptosis in ischemic stroke were not found. This study is to investigate the NVU protective effect and mechanisms of benzoinum on cerebral ischemic rats.

Methods

The neuroprotective activity of benzoinum against MCAO induced cerebral ischemic injury. Neurological scores, TTC staining, HE staining were conducted to evaluate neurological damage. Infarction rate and DCI were calculated. The ultrastructure of neuron and BBB was observed by TEM. Immunohistochemistry and RT-PCR were used to detect the Bax, Bcl-2, Caspase 3 expression. In addition, Claudin 5 also was detected by immunohistochemistry.

Results

The findings shown that benzoinum could significantly improve the neurological function score, reduce the cerebral infarction rate and DCI. Furthermore, benzoinum alleviated pathomorphological change and apoptosis in brain tissue of MCAO rats. The results of TEM and claudin 5 expression of immunohistochemistry showed that benzoinum could play a neuroprotective effect in NVU. Besides, benzoinum enhanced Bcl2, reduced Bax and Bax/Bcl-2, Caspase 3, suggesting benzoinum provided neuroprotective effect by inhibited cell apoptosis.

Conclusion

Benzoinum could play a neuroprotective role and regulate apoptosis to repair and stabilize NVU. Our present findings provide a promising medicine for treatment of ischemic stroke therapy.

Background

Ischemic stroke, which is an ischemic disease caused by impediment or deficiency of blood supply to the brain tissue, is the main cause of death and disability among middle-aged and elderly people [1, 2]. And its prevalence tends to be younger. It has been reported that, among 2.3 billion cardiovascular and cerebrovascular patients, ischemic stroke accounted for 66.4% and cerebral hemorrhage 23.4%. The incidence rate is increasing year by year [3]. Given its high rate of incidence, disability, mortality and recurrence, stroke has become a global health concern and brought a heavy burden on the world,

especially in developing countries [4]. Clinically, the treatment of acute stroke is mainly based on thrombolytic therapy. However, its application is limited by the restriction of the best therapy time and severely cerebral hemorrhage complications [5, 6]. Previous studies of stroke had focused on single neurotropic protection. In recent years, the National Institute of Neurology and Stroke (NINDS) have proposed the concept of neurovascular unit (NVU), which emphasized dynamic transmission of signals among blood vessels, cells, and stromas in the brain [7–9]. Acted as the core structure of NVU, the function of the blood-brain barrier (BBB) depended on the interaction among endothelial cells, astrocytes and stromas. It's reported that the disorder of the stromas could form a barrier of signal transmission among cells or cells and stromas [10]. Cerebral ischemia could break the integrity of BBB, which leads to endogenous edema of blood vessels and aggravates neuronal injury [11]. Therefore, more attention paid on the protection of NVU's integrity after its damage provides a new treatment and research idea for diseases like stroke and other related diseases. Given its features of multi-component and multi-target, Chinese medicine might improve neurological function from various ways to protect NVU and demonstrate its advantages in treating stroke.

As one of the commonly used drugs in clinical aromatic sputum, benzoinum has the function of refreshing and activating blood circulation. Pharmacological studies showed that it could be against cerebral ischemia and hypoxia, regulate BBB. It can also be used for anti-inflammation, anti-apoptosis [12]. Our previous study also found that benzoinum could not only defend mice from acute hypoxic injury [13] and effectively preserve PC12 cells with oxygen sugar deprivation [14], but also obviously prolong the life-span of mice with acute cerebral ischemia [15] and open BBB of mice under physiological conditions [16], which suggested that benzoinum had anti-ischemic effects. The studies showed that apoptosis played an important role in the process of repairing ischemic brain injury and neurovascular homeostasis [17–20]. Therefore, the scientific problem this paper aims to discuss is that whether cerebral ischemia protection of benzoinum is related to apoptosis and neurovascular units remodeling. In this study, the therapeutic effect of benzoinum was explored in middle cerebral artery occlusion (MCAO) rats, and clarify the underlying molecular mechanism. The research process is shown in Fig. 1.

Methods And Material

Animals

Healthy adult male Sprague-Dawley rats weighing 230 to 270 g, were provided by Institute of Experimental Animals, Sichuan Academy of Medical Sciences, and certificate number: SCXK (Chuan) 2013-15. They were housed at the Experimental Animal Center of Chengdu University of Traditional Chinese Medicine.

Drugs

Benzoinum was purchased from the Lotus Pond Chinese herbal medicine market, Chengdu. It was identified by the professor Li Min (Chengdu University of Traditional Chinese Medicine), which comes

from dry resin of *Styrax tonkinensis* (Pierre) Craib ex Hart. Nimodipine (Yabao Pharmaceutical Group Co., Ltd.).

Preparation of drugs

Benzoinum 1g/kg solution: Since benzoinum is a powdered Chinese medicine and contains volatile components, not easily soluble in water, it is formulated with 0.2% carboxymethylcellulose-Na (CMC-Na) and 5% tween-80. The concentration of benzoinum is 0.1 g/ml, which was stored in a refrigerator at 4℃ for use.

Nimodipine: 2 tablets of nimodipine (30 mg/tablet) grinded into powder, and added 50 ml water to prepare a drug solution. The concentration of nimodipine is 1.2 mg/ml, stored at 4℃ for use.

Animal groups and administration

The rats were randomly divided into five groups: sham group, model group, vehicle group (5% tween-80 + 0.2% CMC-Na), nimodipine 0.012 g/kg + model group, benzoinum 1 g/kg + model group. The drugs groups were administered by the intragastric administration to the drug solution (10 ml/kg). The sham and model group were subjected to the same daily treatment with saline, and the vehicle group was given 5% tween+0.2% CMC-Na solution, once/d, continuously for 3 days. After administering the drug 30 min, immediately make MCAO surgery. The experimental process is shown in Fig. 2.

MCAO surgery and treatment

Rats were administrated with 350 mg/kg chloral hydrate intraperitoneally to induce deep general anesthesia prior to surgery. Shave the cervical region and place in supine position. An incision was made to expose the bilateral sternocleidomastoid muscles. The left common carotid artery (CCA) and external carotid artery (ECA) were carefully separated by blunt dissection. A careful separation of the bifurcation of the distal CCA exposed the bifurcations of the ECA and internal carotid artery (ICA). Ligate the bifurcations and CCA close to its proximal end. ICA was clamped with a microscopic clip, and an 18-20mm thread embolism (diameter: 0.286 mm) was inserted into the ICA and fixed in place to the vessel. The sham group only ligated vascular, not thread embolism. 23.5 hour after ischemia, agents were administrated intravenously with correspondent drugs.

Neurological function score

The neurological function scores of each rat were performed after ischemia 4 and 24 h according to Zea Longa [21]. The scores standard is as follows: 0, no signs of neurological deficits; 1, cannot stretch front paws; 2, walking to the uninjured side; 3, circle to the uninjured side; 4, no autonomous walking, a disorder of consciousness.

TTC staining

Rats were anesthetized with chloral hydrate and sacrificed on 1d after operation (n=5 each). Brains of rats were separated and stored at 4℃ for 15 min. The brain tissue was cut into continuous 5 pieces (2 mm), incubated in 2% TTC solution for 30 min. Wash them by PBS and fix in 4% paraformaldehyde (PFA) overnight. Infarcted areas appeared as unstained tissue while normal brain tissue stained red.

HE staining

HE staining was performed to observe the neuronal pathological changes. Rats were anesthetized with chloral hydrate and sacrificed on 1d after operation (n=6 each). Brains of rats were separated and fixed in 4% PFA. dehydrated through graded alcohol and embedded in paraffin wax. Finally, a series of sections (5 μm) was cut by microtome. The sections were prepared for further hematoxylin and eosin (HE) staining Brain tissue staining sections were taken using a Nikon 55i image acquisition system. Each section was counted by taking two non-overlapping representative fields under the microscope, and the cell average value was taken. The degree of injury was expressed as denatured cell index (DCI).

DCI = number of denatured cells / total number of cells.

Immunohistochemistry

Immunohistochemistry was used to detect the expression of Bax, Bcl-2, Caspase-3 and Claudin 5 proteins in the ischemic penumbra brain of rats. Brain slices were fixed in 3% H_2O_2 and 3% normal goat serum and were incubated with Bax, Bcl-2, caspase 3 and Claudin 5 rabbit polyclonal antibodies (1:100, Santa Cruz Biotechnology) in 0.01 mol/L phosphate-buffered saline overnight. The secondary antibodies were from the Vect ABC kit (Zhongshan Biology Technology Company, China). Each slice was selected for 3 fields under a 400-fold microscope, and taken photos. A quantitative average optical density analysis was performed.

Ultrastructure

TEM was performed to observe the ultrastructure pathological changes of neuron and BBB. Rats were anesthetized with chloral hydrate and sacrificed on 1d after operation (n=3 each group). A small piece of ischemic side penumbra brain tissue (1 mm × 1 mm × 1 mm) was cut with a sharp blade, further fixed by 2.5% glutaraldehyde. The histopathological changes in ultrastructure were recorded using transmission electron microscopy (Hitachi HT7700) after staining.

RT-PCR

RT-PCR was used to detect Bax, Bcl-2, Caspase-3 and Claudin 5 gene expression 24 h after the pMCAO (n=3 each group). Total RNA was extracted from the ischemia penumbra in the ipsilateral hemisphere using TriPure Isolation Reagent according to the manufacturer's protocol. Reverse transcription was performed with 2.0 μg of total RNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas International Inc, Burlington, Canada). Forward and reverse primers were used and are shown in Table 1. The reaction system and reaction procedure of RT-PCR were optimized, and factors such as the

amplification curve and the melting curve were determined. The relative expression levels of Bax, Bcl-2, Caspase-3 and Claudin 5 mRNA were calculated using $2^{-\triangle\triangle CT}$.

Table 1 RT-PCR primer sequence

Gene symbol	RefSeq mRNA	Primers
β-actin	NM_031144.3	F: ACAACCTTCTTGCAGCTCCTC
		R:CTGACCCATACCCACCATCAC
Claudin 5	NM_031701.2	F: CTACAGGCTCTTGTGAGGACTTGAC
		R:AGTAGGAACTGTTAGCGGCAGTTTG
Bax	NM_017059.2	F: GTCCAGTTCATCGCCAATTC
		R:CAGGATCGAGCAGAGAGGAT
Caspase-3	NM_012922.2	F: GGGTGCGGTAGAGTAAGCAT
		R:ACAGAGCTGGACTGCGGTAT
Bcl-2	NM_016993.1	F: ACAGCCAGGAGAAATCAAACA
		R:GGTGGACAACATCGCTCTG

Statistical analysis

All experimental data were statistically analyzed by SPSS 19.0 software, expressed as the mean ± standard deviation (SD). Rat neurological function scores were tested by Wilcoxon method, and the other indicators were analyzed by a one-way analysis of variance (ANOVA). The differences were considered statistically significant when $P < 0.05$ and highly significant when $P < 0.01$.

Results

Neurological function score

The neurological function scores were evaluated at ischemia 4 and 24 h, and the results are shown in Fig. 3. Compared with the sham group, the model and vehicle group were significantly increased at 4 and 24 h ($P<0.01$), suggesting the model was successful. Compared with the model group, there was no significant difference in the vehicle group at 4, 24h ($P>0.05$), however, the scores of nimodipine group were significantly decreased ($P<0.01$). Compared with the vehicle group, benzoinum could significantly decrease the scores at 24 h ($P<0.05$), but there was no significant difference at 4 h ($P>0.05$).

TTC staining and determination of cerebral infarction rate

TTC staining showed that (Fig. 4), in the sham group, the brain tissue was structurally intact and rose-red, and no infarcted area was found. Whereas there were significant unstained areas in model and vehicle

group. There were different degrees of reduction in brain infarction areas in animals treated with nimodipine or benzoinum. The results of infarction rate showed that, in the model and vehicle group, the cerebral infarction rate was significantly higher than that of the sham group (P<0.01), indicating that the model was successful. Compared with model group, the cerebral infarction rate was significantly decreased in the nimodipine group (P<0.01). Compared to the vehicle group, the cerebral infarction rate was significantly reduced in benzoinum group (P<0.01).

HE staining

Fig. 5 showed the morphological structure of the brain in each group. Apparent pathological changes occurred in cerebrum tissue of the vehicle group: necrosis, liquefaction, and mesh-like lesions of the nerve cells, which were significantly demarcated from the surrounding tissues. Infarction areas were found in the benzoinum group, whose density was higher. More capillaries were seen in the border area, whose cell density was higher than that in the vehicle group, and new capillary characteristics were observed. In addition, the number of glial cells increased, and abnormal red neurons were less. The DCI of the vehicle group was significantly higher than that of the sham group (P<0.01). The DCI in the benzoinum group was significantly lower than that of the vehicle group (P<0.01).

Ultrastructure

Ultrastructure of neurons

As shown in Fig. 6 a, there is intact and clear nuclear membrane structure, major euchromatin and normal structural organelles in neuron of sham group., there exists dissolved nucleus, incoherent nuclear membrane and less heterochromatin in vehicle group. In the benzoinum group, the nuclear membrane was intact and clear, the heterochromatin in the nucleus was concentrated. There are still many vacuoles, and the organelles were seen in the cytoplasm.

Ultrastructure of BBB

As shown in Fig. 6b, in sham group, the capillary was clear, the basal layer, endothelial cells and glia were close. In vehicle group, there is an edema between the endothelial cells and the basement membrane, the capillary wall was thickened, and the electron density was lowered. In the benzoinum group, the capillary structure was clear, the basal layer was in close contact with the endothelial cells. It indicated that benzoinum could improve the ultrastructure of NVUs.

RT-PCR

Bax, Bcl-2, caspase-3 and claudin 5 mRNA were detected by RT-PCR. The results are shown in Fig. 7. Compared to the sham group, Bax was increased and Bcl2 was decreased. Compared vehicle group, Bcl 2 was significantly increased in benzoinum group (P<0.05), however, Bax was still enhanced in the benzoinum group. Interestingly, Bax/Bcl2 was obviously increased in the vehicle group (P<0.01), while it was significantly decreased in the benzoinum group (P<0.01). In addition, Caspase 3 was significantly

increased in the vehicle group than that of sham group(P<0.01). Benzoinum had a trend to downregulation of Caspase 3 compared to the vehicle group. Meanwhile, benzoinum could significantly up-regulate claudin 5 mRNA expression.

Immunohistochemistry

Bax, Bcl-2, caspase-3 and claudin 5 were quantitated by immunohistochemistry. As shown in Fig. 8, the positive expression of Bax, Bcl-2, caspase-3 and claudin 5 proteins in the ischemic penumbra brain of each group was yellow-brown. Compared with the sham group, Bax was significantly increased (P<0.01) and Bcl2 had a trend of downregulation. What's more, the value of Bax/Bcl-2 were significantly increased in the vehicle group (P<0.01). Compared with the vehicle group, benzoinum could significantly up-regulate Bcl-2 expression (P<0.01), decrease the Bax/Bcl-2 value (P<0.01), and there was no significant difference but had a trend of downregulation of Bax expression (P>0.05). The results of claudin 5 and caspase 3 were shown in Fig. 9. Caspase-3 expression in the ischemic penumbra brain was significantly higher in the vehicle group than that in the sham group (P<0.01). Benzoinum could significantly reduce caspase-3 expression (P<0.01). In addition, benzoinum could enhance claudin 5 expression compared with vehicle group (P<0.01). These findings implied that benzoinum prevention and treatment effectively attenuated ischemic induced cell apoptosis to repair BBB function.

Discussions

Pathological mechanism after stroke is complicated. In recent years, NVU has emerged as a new potential target for understanding the occurrence and development of brain disease [22–25]. In stroke, the function of BBB and the characteristics of neuron are important for repairing injury. Therefore, it is necessary to looking for promising target as a therapeutic approach in stroke [26–28].

Benzoinum is one of the common drugs for clinical aromatic drugs. In modern pharmacological research, benzoinum was antibacterial effect, against ischemic injury, and others [15, 16]. However, there is still no further study on the mechanism of benzoinum in blood vessel, nerom cell apoptosis and the stabilization of NVU. Cerebral ischemia could cause different degrees of neurological dysfunction, such as hemiplegia, limb numbness, hemianopia, and aphasia. Therefore, the evaluation of neurological function draws much attention to doctors and researchers. In this experiment, the results showed that benzoinum could significantly decrease the neurological function score in ischemia rats for 24 h, suggesting that benzoinum could improve the neurological function. Moreover, the cerebral infarction size was evaluated. Our study showed that benzoinum could significantly reduce cerebral infarction size compared with vehicle group. The observations of TTC staining were consistent with the rate of cerebral infarction. To a certain extent, benzoinum played a protective role against cerebral ischemia injury.

After focal cerebral ischemia, the neurons of the central ischemia region appear to be irreversibly damaged [29]. The cells in the ischemia area are loosely arranged and irregular in morphology, and there is a separation of the neurons from the surrounding interstitium, a disappearance of cytoplasmic Nissl bodies and nuclear condensation [30, 31]. The HE staining suggested that pretreatment with benzoinum

could improve the structure of neurons and the number of blood vessels. Benzoinum reduced the DCI of rats induced by MCAO. It was indicated that benzoinum reduced the brain damage by improving the pathological situations in the brain tissue.

The tight junctions are highly developed and played a key role in the establishment of BBB [32, 33]. Claudin 5 is the has been considered the important adhesion molecule of tight junctions involved in BBB in present study [34–36]. In our study, the results suggested that benzoinum could reduce the BBB injury by increasing in Claudin 5. In addition, the ultrastructure of BBB and neuron also revealed benzoinum could protect the structure of NVU and BBB function.

Apoptosis is an important mechanism of NVU homeostasis regulation [37–39]. Bax is a pro-apoptotic signal molecule, while Bcl-2 is an anti-apoptotic signal molecule. In addition, the Bax/Bcl-2 ratio is considered a switch for determining cell apoptosis [40]. They kept a balance in determine the survival of cells [41, 42]. Caspase-3 is the most important apoptosis-executing protease in the process of apoptosis [43, 44]. In recent years, it has been found that Bax and Bcl-2 acted as an upstream factor of Caspase-3 [45, 46]. In our study, it showed that benzoinum increased Bcl-2, decreased caspase-3, and modulating the Bax/Bcl-2, suggesting benzoinum had anti-apoptotic ability. In a word, this finding indicated that benzoinum might inhibit apoptosis in neurons and may maintain the integrity of NVUs (Fig. 10).

Conclusions And Prospects

Benzoinum might reconstitute NVU after MCAO injury by inhibiting apoptosis-related factors to exert the therapeutic effect of cerebral ischemic injury. Benzoinum is a multi-component, multi-target drug. Furthermore, its effective material basis remains to be further studied. And, the deeper mechanism of apoptosis need to be further verified by experiments.

Abbreviations

DCI, denatured cell index; BBB, blood-brain-barrier; NVU, neurovascular unit; MCAO, middle cerebral artery occlusion; TTC, 2,3,5-triphenyl tetrazolium chloride; CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; PFA, paraformaldehyde; SD, standard deviation; CMC-Na, carboxymethylcellulose-Na; HE, hematoxylin-eosin; TEM, transmission electron microscopy; ANOVA, a one-way analysis of variance.

Declarations

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All experimental procedures were carried out according to the protocols approved by Institutional Animal Ethics Committee of Affiliated Hospital of Chengdu University of Traditional Chinese Medicine, with the examination approval No. 2019DL-002.

Consent for publication

Not applicable.

Competing interests

The authors declared that they have no competing interests.

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Figures

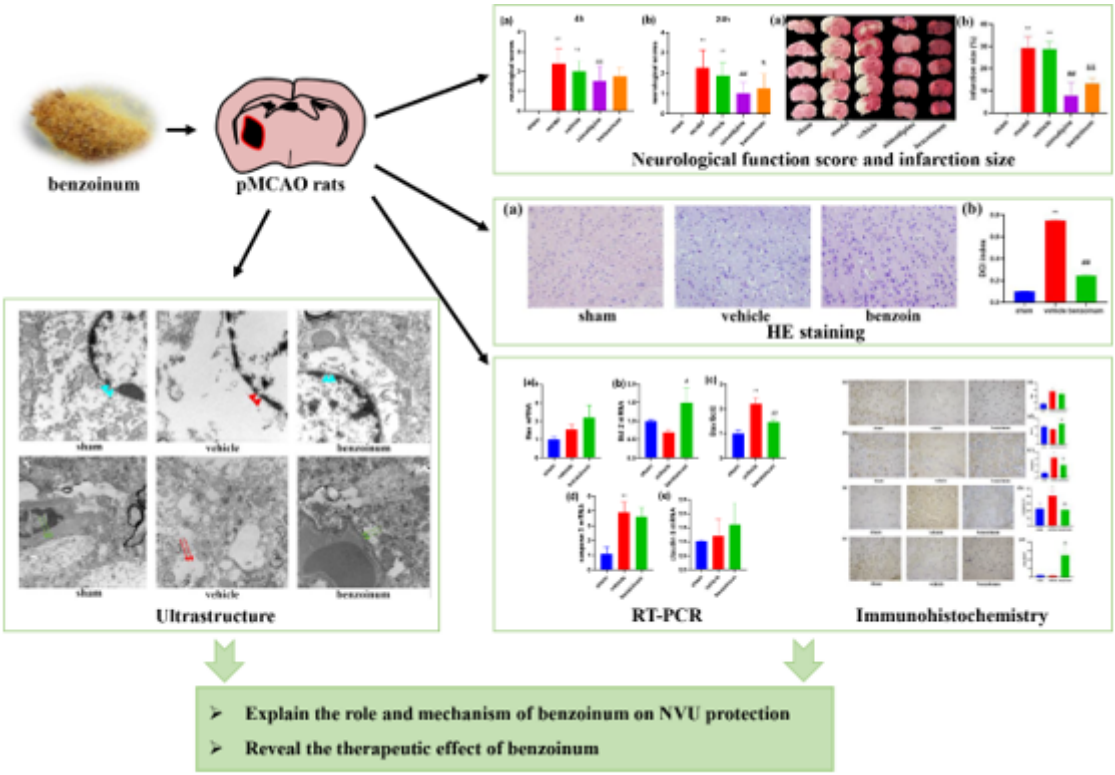

Figure 1

Graphical abstract of this study.

Figure 2

The process of this study.

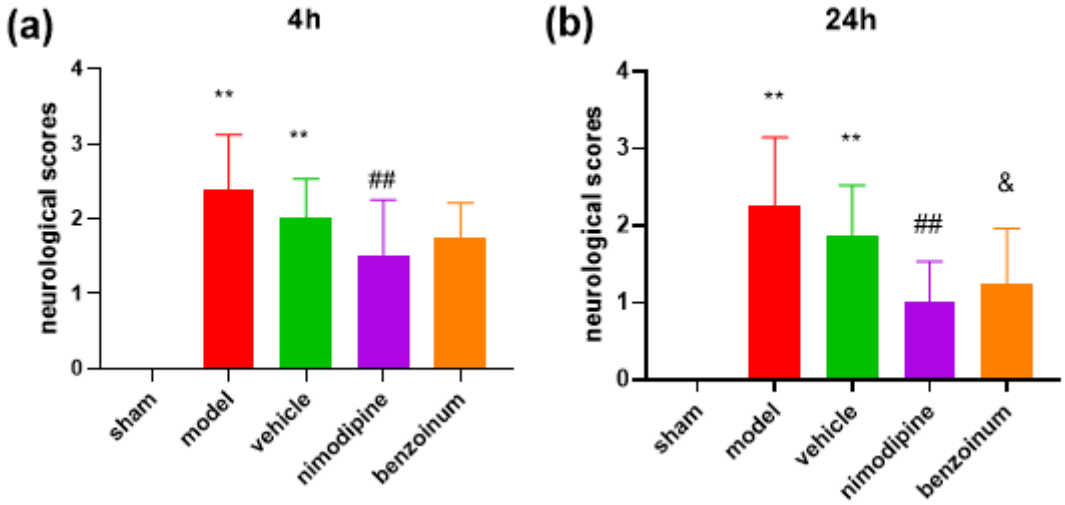

Figure 3

Effects of benzoinum on neurological function score (n=8 each group). Data represented the mean ± SD. (a) 4h after ischemia, (b) 24h after ischemia. **P<0.01 differs from sham group; ##P<0.01 differs from model group; &P<0.05 differs from vehicle group.

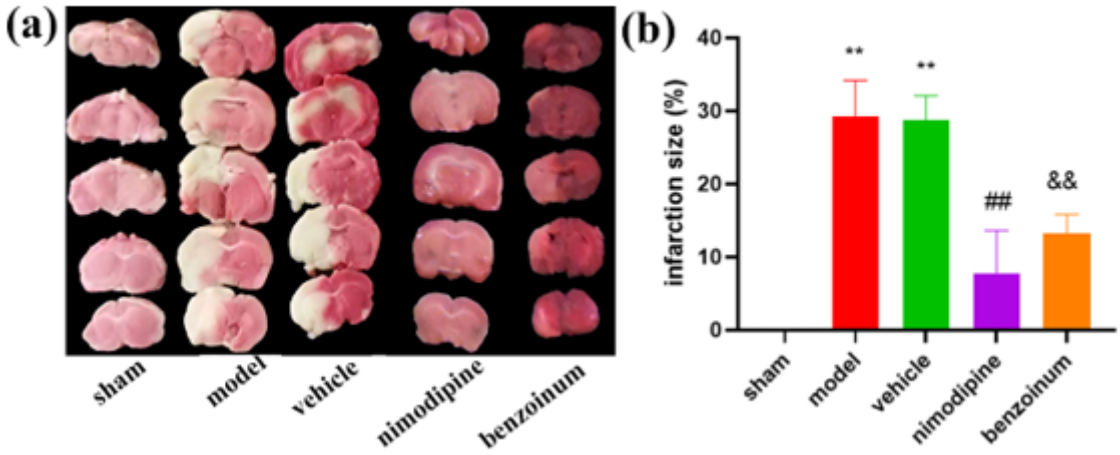

Figure 4

Benzoinum reduced the cerebral infarction size (n=5 each group). (a) TTC staining, (b) infarction size. Data represented the mean ± SD. **P<0.01 differs from sham group; ##P<0.01 differs from model group; &&P<0.01 differs from vehicle group.

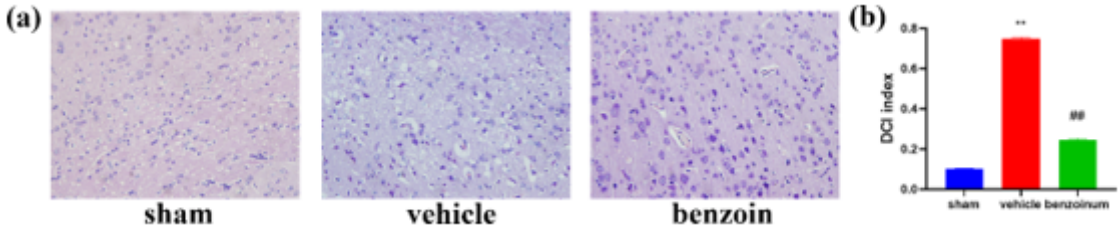

Figure 5

Benzoinum improved pathological changes (n=6 each group). (a) HE staining, (b) DCI index. ** P<0.01 differs from sham group; ##P<0.01 differs from vehicle group.

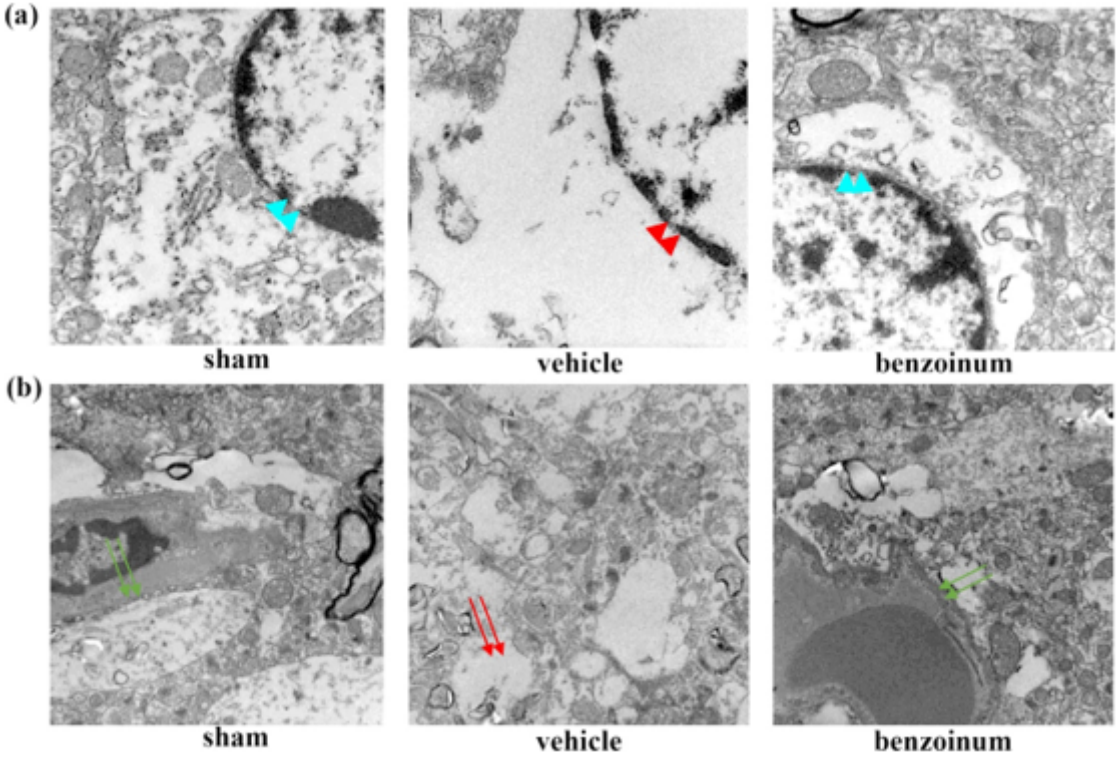

Figure 6

Ultrastructure of neuron and BBB was observed by transmission electronic microscopy. (a) neuron 15000×, (b) BBB 10000×.

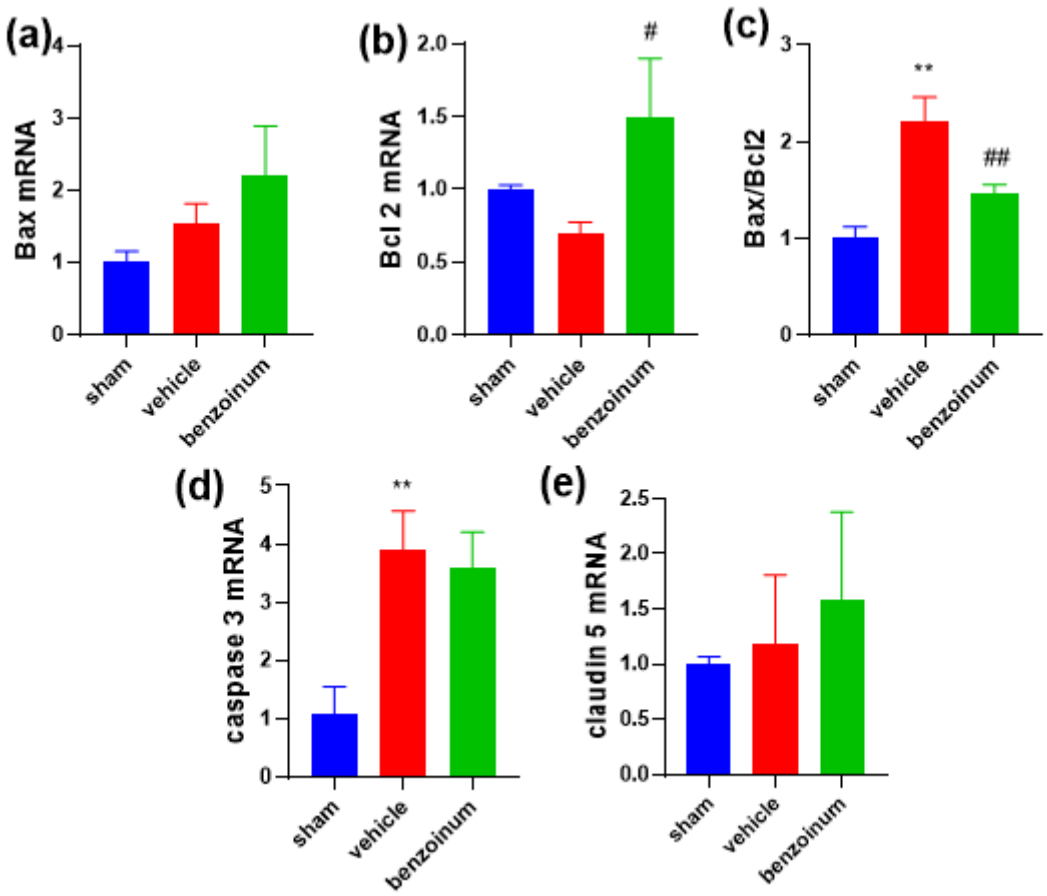

Figure 7

Bax, Bcl-2, caspase-3 and claudin 5 mRNA expression assayed by RT-PCR (n=3 each group). (a) Bax mRNA, (b) Bcl2 mRNA, (c) Bax/Bcl2, (d) caspase 3, (e) claudin 5. * P<0.05 differ from sham group; ##P<0.01 differs from vehicle group.

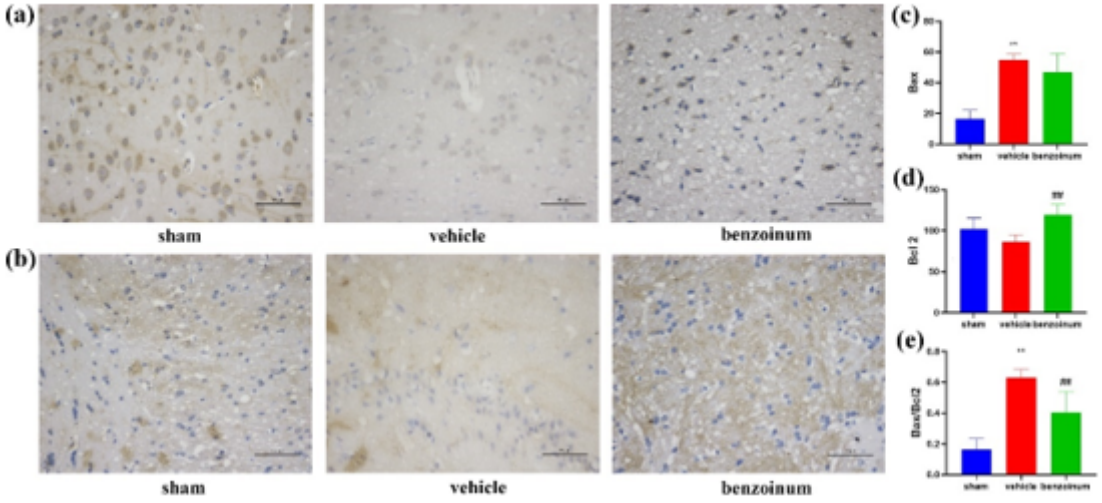

Figure 8

Bax and Bcl-2 assayed by immunohistochemistry. n=6 each group. (a) Bax, (b) Bcl 2, (c) the anslysis of Bax, (d) the analysis of Bcl2, (e) Bax/Bcl2. ** P<0.01 differ from sham group; ##P<0.01 differs from vehicle group.

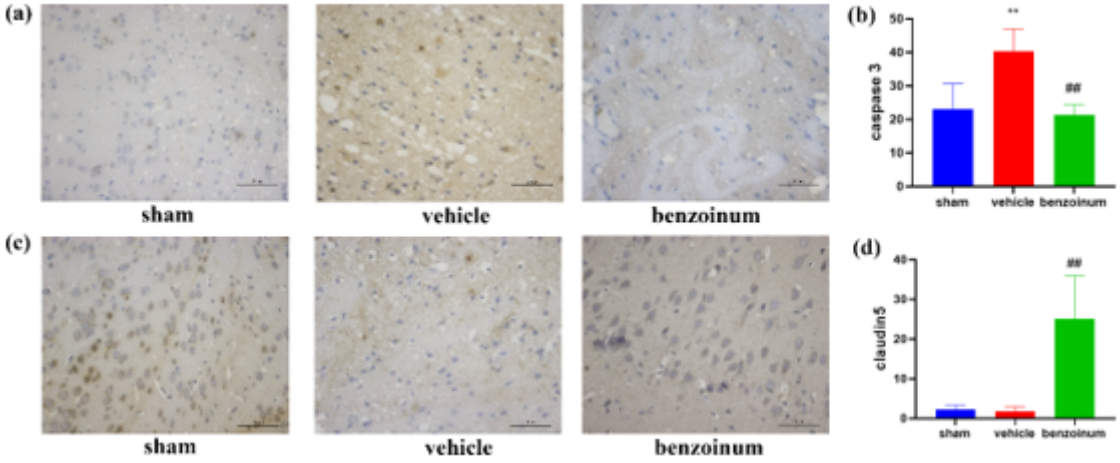

Figure 9

Caspase 3 and Claudin 5 assayed by immunohistochemistry. n=6 each group. (a) Caspase 3, (b) the analysis of Caspase 3, (c) Claudin 5, (d) the analysis of Claudin 5. ** P<0.01 differ from sham group; ##P<0.01 differs from vehicle group.

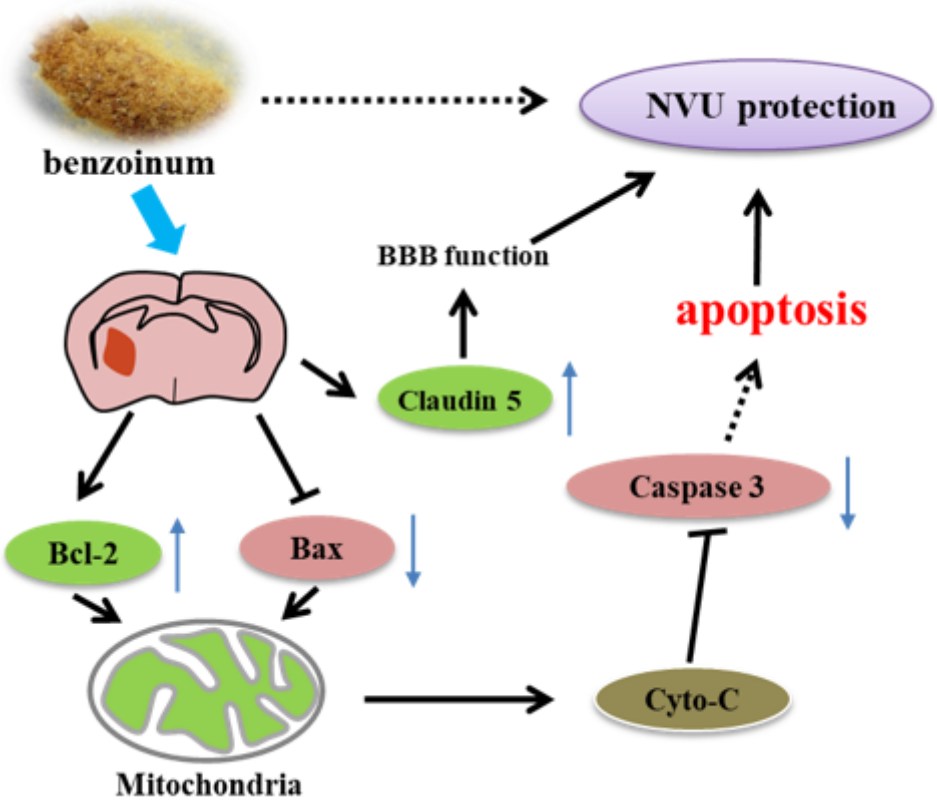

Figure 10

The apoptosis mechanisms of benzoinum on NVU protection.