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## Improvement in the Ultrastructures of Nervous Tissues Damaged in Cerebral Ischemic Rate by Picroside II

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### ABSTRACT

The aim of present study was to verify the neuroprotective effect of picroside II on cerebral ischemic injury in rats from the perspective of neuropathology. Healthy adult male Wistar rats were used to establish the forebrain ischemic model. Transmission Electron Microscopy (TEM) was used to observe the ultrastructure of neurons, astrocytes, myelin and the Blood-Brain Barrier (BBB). There was morphological damage in neuron, astrocyte, myelin and BBB after cerebral ischemia. After treatment by picroside II, the degree of damage in neuron, astrocytes, myelin and BBB was reduced. These results suggest that picroside II could significantly improve the morphosis of nerve tissue after cerebral ischemia and play a neuroprotective effect on cerebral ischemic injury in rats.

**Key words:** Picroside II, cerebral ischemia, ultrastructure, transmission electron microscopy, rats

### INTRODUCTION

Stroke is the second main reason of the death and disability for adult (Heron, 2011), among which ischemic stroke accounts for 60-80%. Ischemic stroke mainly involves the increased excitatory amino acids, oxidative stress, inflammation, calcium overload and other pathological processes, eventually leading to cell necrosis or apoptosis (Caso *et al.*, 2008; Bi *et al.*, 2009). Ultra-early thrombolytic therapy (<3 h) has been considered the most important measures to restore the blood supply of brain at early stage (Wahlgren *et al.*, 2007). However, most patients lose the opportunity of ultra-early thrombolysis due to the restriction of medical condition. So, it is very important to explore the therapeutic method to extend the treatment time window. Previous animal experiments indicated that many neuroprotective agents had certain therapeutic effect while their clinical application remained to be confirmed (Stavchanskii *et al.*, 2013; Liu *et al.*, 2013). Traditional Chinese Medicine had a long history in the treatment of stroke and the therapeutic effect was reliable (Wu *et al.*, 2011) but its mechanism of treatment is not clear yet. Some clinical trials confirmed that Picrorhizae, one kind of Chinese medicine, has effects of heat dampness (Jiangsu New Medical College,

1996). Experimental studies confirmed that picroside II, as the main active ingredient of Picrorhizae, had functions of antioxidant, anti-inflammatory and anti-apoptosis (Li *et al.*, 2007; Cao *et al.*, 2007), as well as reduced the infarct volume and improved the behavior of animals (Li *et al.*, 2010a, b). From the immunohistochemistry and the gene and protein levels, the authors confirmed that picroside II could down-regulate the expression of inflammatory cytokines, such as Toll-Like Receptor 4 (TLR4), Nuclear Factor  $\kappa$ B (NF $\kappa$ B), caspase enzymes-3 (caspase-3) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), thereby inhibit neuronal apoptosis induced by ischemia (Guo *et al.*, 2010; Li *et al.*, 2010c) and play an important role in the protection of brain (Pei *et al.*, 2012). Although modern molecular biological indicators have high sensitivity and specificity in the detection of cerebral ischemic injury, the classical histopathological and ultrastructural morphological indicators are still the most credible objective indicators to evaluate the neuronal damage and recovery and promoting the recovery of neurological structure and function is also the ultimate goal of various therapeutic methods. This study aimed to validate the protection effects of picroside II in cerebral ischemia by observing its influence on the morphology and structure of brain tissue.

## MATERIALS AND METHODS

**Establishment of animal models:** Total of 35 adult healthy male Wistar rats (weighted 230-250 g, SPF grade) were supplied by the Experiment Animal Center of Qingdao Drug Inspection Institute (SCXK (LU) 20100100). All animals were acclimatized for 7 days and allowed to take food and water freely at room temperature ( $23\pm 2^{\circ}\text{C}$ ) and humidity-controlled housing with natural illumination and fasting for 12 h before operation. Ten rats were randomly selected for control group and the rest 25 rats were anesthetized by injecting intraperitoneally 10% chloral hydrate ( $3\text{ mL kg}^{-1}$ ) and fixed in supine position to conduct aseptic operation strictly. The forebrain ischemic models were established by Bilateral Common Carotid Artery Occlusion (BCCAO) (Marquez-Martin *et al.*, 2012). Core body temperature was keeping with a rectal probe and maintained at  $36-37^{\circ}\text{C}$  using a homeothermic blanket control unit during and after the surgery operation and laser doppler flowmetry (PeriFlux 5000, Sweden) was used to continuous monitor the Cerebral Blood Flow (CBF). Five rats un-waked or died after 1.5 h of operation were rejected while 20 successful model rats which CBF curve dropped to 30% were brought into the experiment and randomly divided into model group and treatment group ( $n = 10$ ). The rats of control group were operated as the same experimental procedures besides BCCAO. This experiment was approved by the Ethics Committee of Qingdao University Medical College (QUMC 2011-09). The local legislation for ethics of experiment on animals and guidelines for the care and use of laboratory animals were followed in all animal procedures.

**Intervention:** Picoside II (molecular formula:  $\text{C}_{23}\text{H}_{28}\text{O}_{13}$ , molecular weight: 512.48 g, CAS No: 39012-20-9, purity >98%) supplied by Tianjin Kuqing Med. Tech. Co., Ltd. and was diluted to 1% solution by normal saline. According to preliminary studies which we found the optimal therapeutic dose and time window (Zhao *et al.*, 2014), the treatment group animals were injected intraperitoneally picoside II  $20\text{ mg kg}^{-1}$  body weight after ischemic 1.5 h while saline were injected intraperitoneally in control group and model group after ischemic 1.5 h.

**TEM:** Took five animals in each group and anesthetized with 10% chloral hydrate ( $3\text{ mL kg}^{-1}$ ) after treatment 24 h by picoside II, then removed the brains from the parietal cortex of the ischemic area following perfusing with normal saline 200 mL and cut into small pieces of  $1\times 1\times 1\text{ mm}$  and placed quickly in 2.5% glutaraldehyde for 24 h. Then rinsed  $15\text{ min}\times 3$  times by  $0.1\text{ mol L}^{-1}$  phosphate buffer and rinsed again  $15\text{ min}\times 3$  times by  $0.1\text{ mol L}^{-1}$  phosphate buffer after fixing 2 h by 1% osmium tetroxide fixative; dehydrated by acetone as the following gradient: 50% acetone 15 min, 70% acetone 15 min, 80% acetone 15 min, 90% acetone 15 min, 100% acetone  $10\text{ min}\times 2$  times; then soaked at  $37^{\circ}\text{C}$  for 1.5 h in mixture (acetone: package buried solution = 1:1) and soaked overnight in pure embedding liquid at  $37^{\circ}\text{C}$  and the sample was embedded into an epoxy resin plate in Epon812, putted into the oven for polymerization 12 h at  $37, 45^{\circ}\text{C}$  12 h,  $60^{\circ}\text{C}$

48 h and finally embedded into block. Put the paraffin block on a dissecting microscope with a special gripper, then made into cone shape after chip-off the embedding medium with a sharp blade. Ultramicrotome (Leica EM UC6, Germany) slice thickness of 50 nm and placed on Formvar film copper grid, stored at  $4^{\circ}\text{C}$ .

Dripped a drop of 3% uranyl acetate-alcohol saturated solution ( $\text{pH} = 3.5$ ) in a petri dish, covered the nets of ultrathin sections to contact with the dye liquor to stain for 30 min and rinsed with double-distilled water for  $10\text{ min}\times 3$  times to suck up water. Then, covered the nets of ultrathin sections to a drop of 6% lead citrate dye liquor ( $\text{pH} = 12$ ) in another petri dish to stain for 5 min, rinsed with non-carbon dioxide double-distilled water for  $10\text{ min}\times 3$  times, dried at room temperature. The ultrastructure of neurons, glial cells, myelin and blood-brain barrier was observed under TEM (JEM-1200EX, Japan).

## RESULTS

**Ultrastructure of neurons:** In control group, neuron had clear double membrane structure, mitochondria was rich and elongated oval and had intact structure of endoplasmic reticulum, golgi apparatus, lysosomes and other organelles, the nuclear membrane nucleus, nucleolus, nuclear pore were clear, chromatin were well-arranged and distributed evenly and orderly, had a high electronic permeability (Fig. 1a-b). In model group, cell morphology was irregular, double membrane structure was incomplete, organelles were broken or lost, nuclear condensation, nuclear membrane damage and chromatin condensation (Fig. 1c-d). Compared with model group, neuronal injury improved significantly in treatment group, the number of mitochondria increased and the aggregation of chromatin and cytoplasmic vacuoles reduced (Fig. 1e-f).

**Ultrastructure of astrocytes:** In control group, the structure of astrocytes was integrity, membrane structure had a clear outline, organelle structure was well and chromatin was well-distributed (Fig. 2a-b). In model group, astrocytes were complete while organelles were broken or lost, there were a large number of vacuoles in cytoplasm, nuclear shrinkage, chromatin condensed and uneven distribution were found in nucleus (Fig. 2c-d). After treatment, the injury of cells improved significantly than that in the model group, chromatin was lightly stained, mitochondrion increased and vacuoles decreased (Fig. 2e-f).

**Ultrastructure of nerve myelin:** In control group, myelin fibers were regular structure, axonal clear and had the intact mitochondria (Fig. 3a-b). In model group (Fig. 3c-d), myelin hierarchy was unclear and merging, irregular, axonal disappeared, showing vacuolization. After treatment (Fig. 3e-f), myelin fiber damage improved significantly compared with model group, myelin sheath and neuraxon became distinct.

**Ultrastructure of blood brain barrier:** In control group (Fig. 4a-b), vascular endothelial cells were complete,

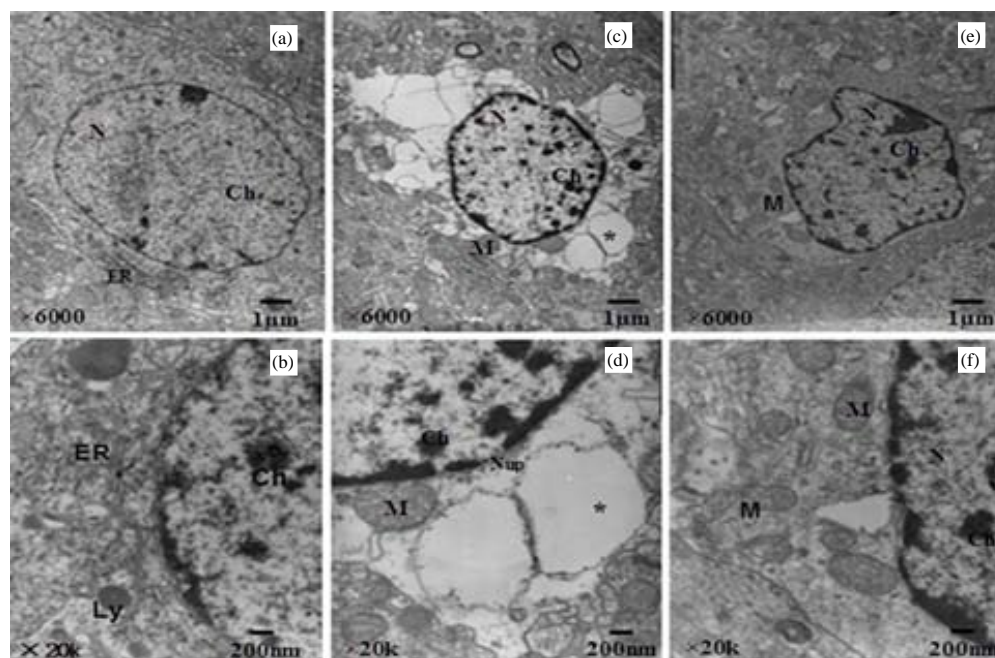


Fig. 1(a-f): Ultrastructure of neuron by TEM, (a-b) Control group, (c-d) Model group and (e-f) Treatment group; N: Nucleus, Nup: Nuclear pore, Ly: Lysosomes, ER: Endoplasmic reticulum, Ch: Chromatin, M: Mitochondrion, \*Cytoplasmic vacuoles

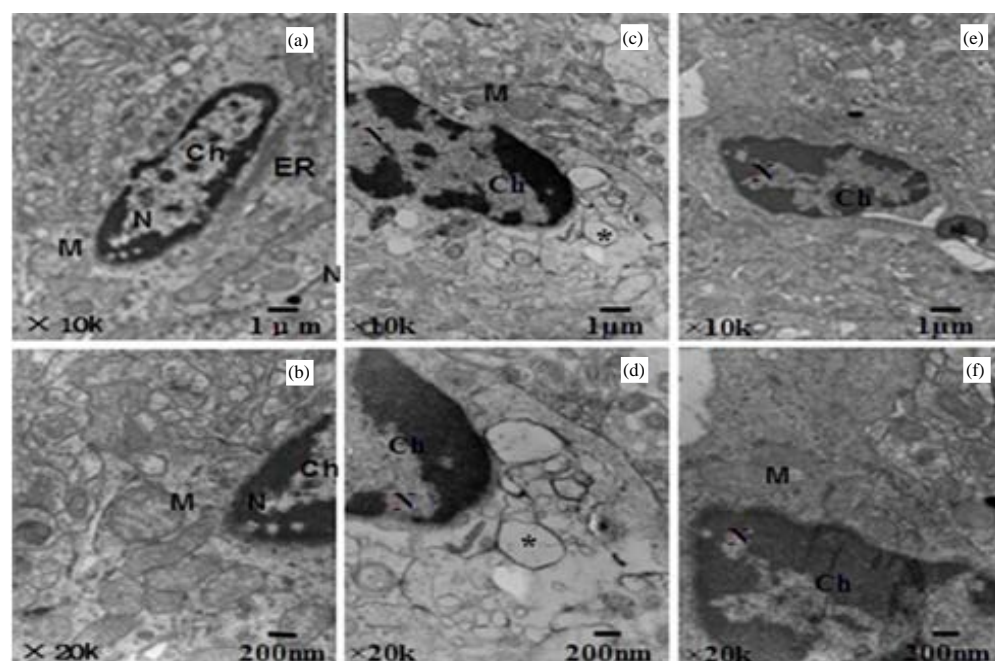


Fig. 2(a-f): Ultrastructure of astrocyte by TEM, (a-b) Control group, (c-d) Model group and (e-f) Treatment group, N: Nucleus, ER: Endoplasmic reticulum, Ch: Chromatin, M: Mitochondrion, \*Cytoplasmic vacuoles

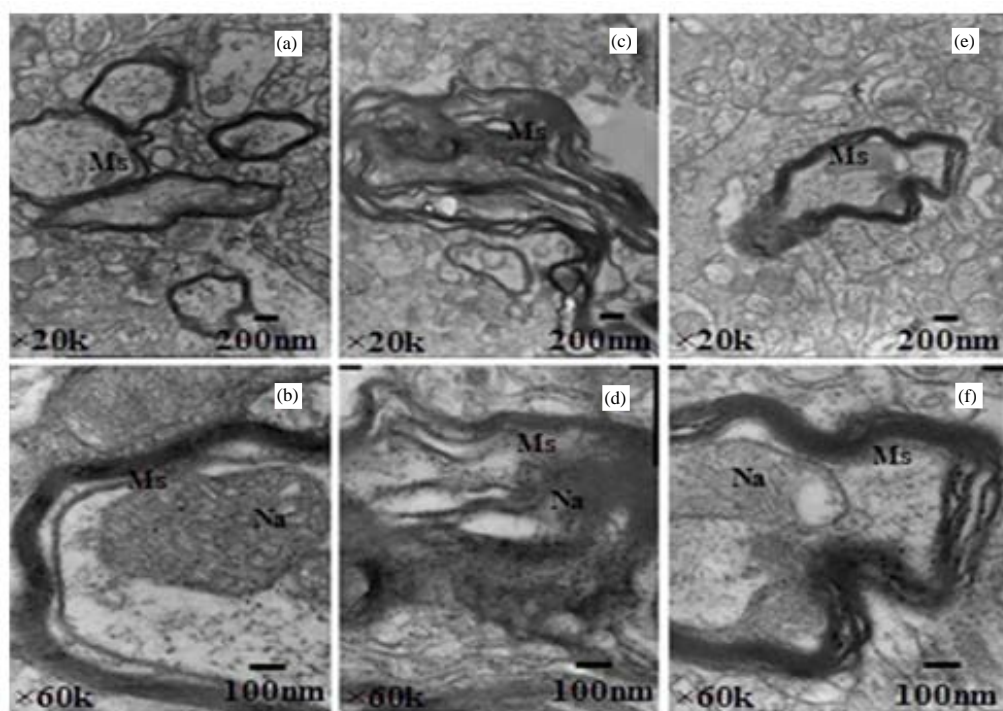


Fig. 3(a-f): Ultrastructure of nerve fibers myelin by TEM, (a-b) Control group, (c-d) Model group and (e-f) Treatment groups, Ms: Myelin sheath, Na: Neuraxon

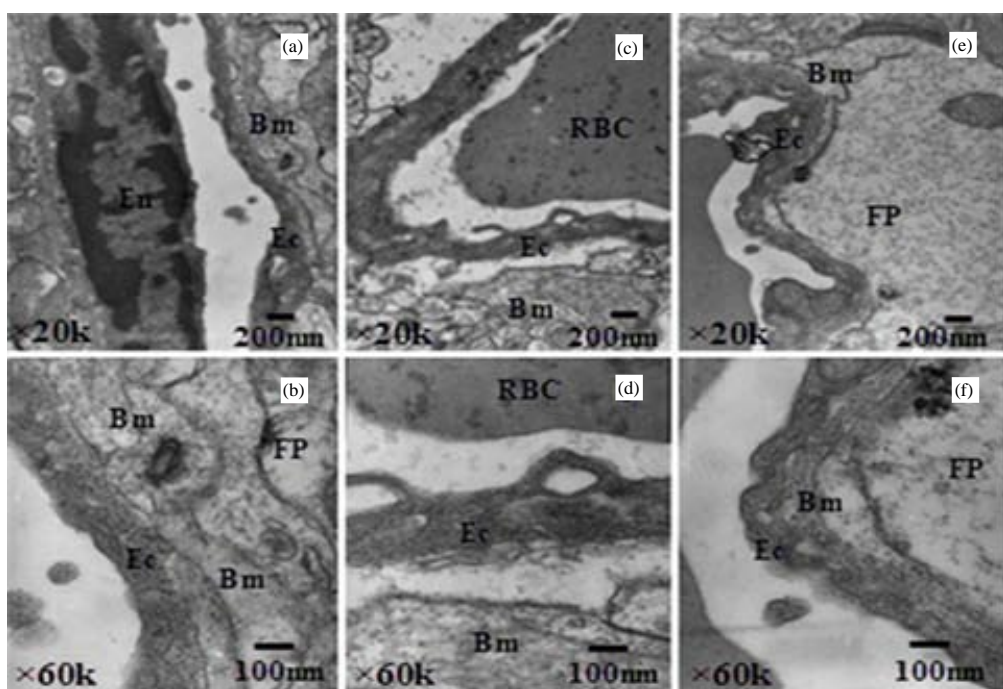


Fig. 4(a-f): Ultrastructure of blood brain barrier by TEM, (a-b) Control group, (c-d) Model group and (e-f) Treatment group, En: Endothelial nucleus, Bm: Basement membrane, Ec: Endothelium cell, FP: Foot process, RBC: Red blood cell



endothelium was smooth, basement membrane and glia foot processes were distinctive nuance. While in model group (Fig. 4c-d), basement membrane and glia foot were separated from endothelium, endothelium was thinner, rough, basement membrane dissolved away, electron density of foot process was decreased. In treatment group, the damage of brain tissue and blood-brain barrier improved significantly compared with model group (Fig. 4e-f), endothelium cell and basement membrane were distinctive nuance.

## DISCUSSION

As a traditional Chinese medicine, Picrorhizae has functions of cleaning heat, drying humidity, retreating steam, cooling blood and cholagogue (Jiangsu New Medical College, 1996) and picroside II is one of its active ingredients. Cell culture confirmed that picroside II could reduce the H<sub>2</sub>O<sub>2</sub>-induced injury in PC12 cells and promote cell survival (Li *et al.*, 2002). Animal experiments (Li *et al.*, 2010a) confirmed that picroside II and tanshinol sodium could reduce the neuronal apoptosis and down-regulate the expression of Caspase-3 and PARP which indicated picroside II has an antioxidant effect similar to tanshinol.

The NSE is the specific marker enzyme of neurons and neuroendocrine cells, as well as the marker enzyme of cerebral ischemic injury (Ahmad *et al.*, 2012). After cerebral ischemic and hypoxic injury, the expression of NSE in brain tissue increased following neurons and myelin damaged, then NSE was released from necrotic cells into cerebrospinal fluid and then into blood passing the damaged blood-brain barrier (Gelderblom *et al.*, 2013). In this experiment, the microstructure of neurons was observed under electron microscopy. In control group, neurons had regular shape, evenly distributed chromatin and organelle with integrity structure; in model group, the membrane of neurons were damaged and the organelle broken or disappeared, proving that there had damaged neurons after cerebral ischemia. After treatment with picroside II, the injury of neurons was mitigated significantly than that in model group, proving that picroside II has protective effects against cerebral ischemic injury.

The S100B exists in astrocytes and Schwann cells of nervous system (Hatfield and McKernan, 1992). It was found that a large number of S100B can promote inflammation and accelerate neuronal apoptosis (Murabayashi *et al.*, 2008). When cerebral ischemia occurs, large amounts of S100B was produced by glia and released into extracellular space (Yardan *et al.*, 2011), then into cerebrospinal fluid and blood passing the damaged blood-brain barrier, leading to a high concentration of S100B in serum which is closely related to the severity, prognosis and mortality of cerebral ischemic injury (Brouns *et al.*, 2010). In this experiment, the morphology of glia was observed under electron microscopy. In control group, the outline of glia and the structure of organelles were complete and nuclear chromatin distributed evenly; in model group, the shape of glia were irregular with organelles broken or even disappeared and a large number of

vacuoles can be observed in cytoplasm. And the number of damaged glia in model group observed under light microscope increased significantly compared with control group. In treatment group, the injury of glia reduced significantly, further proving the protective effects of picroside II in cerebral ischemic injury.

The MBP locates in the serosal surface of myelin and plays an important role on the stability of the structure and function of myelin in central nervous system (Kazmierski *et al.*, 2012). MBP is a specific protein marker for judging the damage of myelin (Wunderlich *et al.*, 2004). Animal experiments showed that there was a small amount of MBP mRNA expressed in brain tissue of normal adult rats and after cerebral ischemic injury the expression of MBP mRNA decreased (Strand *et al.*, 1984). In the present experiment, the results of fast green staining were observed under light microscope and electron microscope. In control group, the myelin distributed evenly and packed tightly under light microscope; and the myelin fibers well arranged, regular structured as well as had the clear axonal under electron microscope. While myelin in model group was loose and disordered arranged, with axon disappeared. Compared with model group, the injury of myelin was alleviated after treated with picroside II. It proved that picroside II played a protective effect on myelin after cerebral ischemic injury.

Blood-Brain Barrier (BBB) consists of microvascular endothelial cells, astrocytes and basement membrane and plays an important role in the maintenance of brain homeostasis. After brain ischemic and hypoxic injury, because of the energy metabolic disturbance and calcium overload which could activate degrading enzyme, the endothelial cells was damaged and membrane permeability increased, finally leading to the open of BBB (Wu *et al.*, 2006). This finding provided that BBB is the material basis for the overflow of biological marker enzyme (such as NSE, S100B, MBP) in brain injury. This study confirmed that endothelial cells of normal BBB were intact, basement membrane and foot processes of glia were distinct. In model group, the BBB was damaged. Compared with model group, the damage of BBB in rats after treated with picroside II was significant with endothelial cells necrosis, basement membrane and glia foot processes dissolved or even disappearantly mitigated. In treatment group, the injury of glia and apoptosis of endothelial cells were reduced, suggesting that picroside II could play an important role in the protection of BBB by reducing the injury of glia and vascular endothelial cells apoptosis.

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