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Research paper

Protective effects of Ephedra sinica extract on blood–brain barrier integrity and neurological function correlate with complement C3 reduction after subarachnoid hemorrhage in rats

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HIGHLIGHTS

- Ephedra Sinica alleviated neurological deficits and cortex cell death after SAH.
- Ephedra Sinica reduced brain edema and blood brain barrier permeability after SAH.
- Ephedra Sinica inhibited the deposition and expression of compliment C3 after SAH.
- Ephedra Sinica increased sonic hedgehog and osteopontin expressions after SAH.
- Ephedra Sinica reduced matrix metalloproteinase-9 expression after SAH.

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ABSTRACT

Early brain injury, which is associated with brain cell death, blood–brain barrier disruption, brain edema, and other pathophysiological events, is thought to be the main target in the prevention of poor outcomes after subarachnoid hemorrhage (SAH). Emerging evidences indicates that complement system, especially complement C3 is detrimental to neurological outcomes of SAH patients. Recently, Ephedra sinica extract was extracted and purified, which exhibits ability to block the activity of the classical and alternative pathways of complement, and improve neurological outcomes after spinal cord injury and ischemic brain injury. However, it is still unclear whether Ephedra sinica extract could attenuate early brain injury after SAH. In the present study, a standard endovascular perforation model was used to produce the experimental SAH in Sprague-Dawley rats. Ephedra sinica extract (15 mg/kg) was orally administrated daily and evaluated for effects on modified Garcia score, brain water content, Evans blue extravasation and fluorescence, cortex cell death by TUNEL staining, and the expressions of complement C3/C3b, activated C3, sonic hedgehog, osteopontin and matrix metalloproteinase-9 by western bolt and immunofluorescence staining. We founded that the Ephedra sinica extract alleviated the blood–brain barrier disruption and brain edema, eventually improved neurological functions after SAH in rats. These neuroprotective effects was associated with the inhibition of complement C3, possibly via upregulating sonic hedgehog and osteopontin signal, and reducing the expressions of matrix metalloproteinase-9. Taking together, these observations suggested complement C3 inhibition by the Ephedra sinica extract may be a protective factor against early brain injury after SAH.

1. Introduction

Subarachnoid hemorrhage(SAH) is a subtype of intracranial hemorrhage with high morbidity and mortality. Early brain injury, which is associated with brain cell death, blood–brain barrier disruption, brain edema, and other pathophysiological events, is thought to be the main target in the prevention of poor outcomes

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post-SAH [1]. Emerging evidences indicates that complement system, especially complement C3 is detrimental to neurological outcomes of SAH patients [7,18,29].

A Chinese herb, Ephedra sinica, is commonly used in anti-inflammatory and autoimmune diseases in Chinese traditional medicine [10]. Recently, Ling M, et al. extracted and purified an Ephedra sinica extract, which exhibited ability to block the activity of the classical and alternative pathways of complement [16]. Therefore, they also named this Ephedra sinica extract as Complement Inhibiting Component of Ephedra Sinica (CICES). Meanwhile, other studies indicated that Ephedra sinica extract could target central nervous system and alleviate acute brain injury, including spinal cord injury [11] and ischemic brain injury [26].

However, it is still unclear whether Ephedra sinica extract could attenuate early brain injury post-SAH. Therefore, we utilized the same Ephedra sinica extract as previous in the present study. We hypothesize that Ephedra sinica extract may alleviated blood–brain barrier disruption and improve neurological function via Inhibiting Complement C3 post-SAH in rats and sought to obtain more direct pieces of evidence for this hypothesis.

2. Materials and methods

2.1. Experimental animals

All protocol involving animal experiments were approved by the Ethics Committee of the Southwest Hospital and performed in accordance with the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

In the present study, one hundred and eighty-five (185) male Sprague-Dawley rats (Experiment Animal Center of Third Military Medical University, Chongqing, China), weighing between 250 and 300 g, were randomly divided into the following groups: Sham + Normal Saline group (Sham + NS, $n = 41$), Sham + Ephedra sinica extract group (Sham + CICES, $n = 28$), SAH + Normal Saline group (SAH + NS, $n = 59$), and SAH + Ephedra sinica extract group (SAH + CICES, $n = 57$).

2.2. SAH model

The endovascular perforation SAH model was induced as our previously study [3]. Briefly, anesthesia was induced with sodium pentobarbital (40 mg/kg, intraperitoneally). The body temperature was kept at $37.0 \pm 0.5^{\circ}$C by using a constant temperature pad during the surgery. Then, the left common carotid artery was exposed under surgical microscope, and sharpened 3–0 nylon suture was inserted into external carotid artery and advanced through intracranial internal carotid artery until resistance was felt. The suture was then pushed 3 mm further to perforate internal carotid artery wall and was subsequently withdrawn into external carotid artery, where internal carotid artery was then reperfused. For sham rats, the filaments were advanced into internal carotid artery, but no arterial perforation was performed.

2.3. Drug administration

Ephedra sinica extract, also named CICES (Jinmai Biotech, Chongqing, China), was dissolved in distilled water at the concentration of 3 mg/ml, and pH value was adjusted to 5.0 with 1 mol/L hydrochloric acid [16]. Immediately after the surgery, Ephedra sinica extract (15 mg/kg) [11] was orally administrated in rats by gavage and was repeated every 24 h until sacrifice. Normal Saline was used as control and administrated in same manner.

2.4. SAH grade

The severity of the SAH was blindly measured at time of the sacrifice on the basis of a grading scale as previously described as SAH Grade [23]. Shortly after removing the brain, an image of the underside of the brain was taken and these images were then divided into 6 parts (left and right frontal, left and right temporal, and upper and lower brain stem). Each of these images was allotted a grade from 0 to 3, depending on the amount of subarachnoid blood that remained within the segment: grade 0, no subarachnoid blood; grade 1, minimal subarachnoid blood; grade 2, moderate blood clot with recognizable arteries; and grade 3, blood clot obliterating all of the arteries within the segment. The rats received a total score, which ranged from 0 to 18 by two blinded observers.

2.5. Neurological scores

The neurological functions were evaluated at 24 h and 72 h ($n = 7$) after the first drug administration, on the basis of Modified Garcia Scale which has been previously described [2]. It consisted of six tests including spontaneous activity, spontaneous movement of four limbs, forepaw outstretching, climbing, body proprioception, and response to whisker stimulation (3–18 points). The mean of neurologic score was evaluated by two blinded observers for grading.

2.6. Brain water content

The brains were quickly separated into left and right cerebral hemispheres, and weighed (wet weight) at 24 h or 72 h ($n = 7$) post-SAH, then weighed again (dry weight) when brain samples were dried. The percentage of brain water content was calculated as (wet weight–dry weight)/wet weight $\times$ 100%.

2.7. Evans blue extravasation and fluorescence

The BBB disruption was evaluated using Evans blue dye extravasation as previously described [28]. Briefly, at 24 h post-SAH, Evans blue dye (2%, 5 ml/kg) was injected and administered over 2 min into the left femoral vein, where it was allowed to circulate for 60 min. Under anesthesia, rats ($n = 7$) were sacrificed by an intracardial perfusion with saline, then brains were removed and divided into left and right cerebral hemispheres to evaluate the Evans blue extravasation. The brain samples were weighed, homogenized in saline, and centrifuged at 15000 g for 30 min. Then, an equal volume of trichloroacetic acid was added to the resultant supernatant. The samples were then incubated overnight at 4 °C and centrifuged at 15000 g for 30 min. The resultant supernatant was then spectrophotometrically quantified for the extravasated Evans blue dye at 620 nm.

For Evans blue florescence, sterile saline was replaced by 4% paraformaldehyde after intracardial perfusion. Then brain specimen ($n = 3$) were removed to be prepared for coronal brain sections (10 μm) as same as immunohistochemistry staining. Then, the red auto-florescence of Evans blue dye was observed on slides using excitation and emission fitters for red florescence (Olympus OX51, Tokyo, Japan).

2.8. TUNEL staining

The brain samples ($n = 3$) were prepared at 24 h and 72 h post-SAH as previously described [14]. Cortex cell death was detected using an In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) and performed according to the manufacturer's instructions, then stained with 3, 3′-diaminobenzidine for illustration.