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# Ursolic acid reduces oxidative stress to alleviate early brain injury following experimental subarachnoid hemorrhage

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## HIGHLIGHTS

- Ursolic acid alleviated early brain injury following subarachnoid hemorrhage.
- Oxidant stress is related to subarachnoid hemorrhage.
- Ursolic acid is an antioxidant.

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## ABSTRACT

Ursolic acid (UA), a well-known anti-oxidative reagent, has been reported to protect the brain against ischemic stoke. However, the potential role of UA in protecting against early brain injury (EBI) after subarachnoid hemorrhage (SAH) remains unclear. The present study aimed to examine the effect of UA against EBI following SAH, and to demonstrate whether the effect is associated with its powerful antioxidant property. Male SD rats were divided into vehicle-treated sham, vehicle-treated SAH, and UA-treated SAH groups. The endovascular puncture model was used to induce SAH and all the rats were subsequently sacrificed at 48 h after SAH. The results show that UA administration could significantly attenuate EBI (including brain edema, blood–brain barrier disruption, neural cell apoptosis, and neurological deficient) after SAH in rats and up-regulate the antioxidative levels in the rat cerebral cortex, suggesting that administration of UA in experimental SAH rats could alleviate brain injury symptom, potentially through its powerful antioxidant property. Hence, we concluded that UA might be a novel therapeutic agent for EBI following SAH.



## 1. Introduction

Subarachnoid hemorrhage (SAH) is a serious condition with high mortality and morbidity [22]. In China, crude annual incidence of SAH was 6.2% for men and 8.2% for women in a study among 226 patients [26]. The therapies for SAH in reducing the incidence of cerebral vasospasm cannot improve long-term neurological outcome, indicating that its importance in patient outcome is misinterpreted. More recently, early brain injury (EBI) has been considered to be the main cause of poor outcomes in SAH patients instead of cerebral vasospasm [15]. EBI refers to the acute injuries to the whole brain encompassing 24–72 h following SAH, while the exact mechanisms of EBI are not fully understood.

Previous studies indicate that oxidative damage plays a critical role in EBI pathogenesis and may become a novel target for treatment of SAH. Substantial evidences [6,16] have demonstrated that the level of oxidative stress significantly increase following SAH, and the effect of antioxidative therapy in experimental SAH and clinical trials is prominent, therefore, the use of free-radical scavengers is a reasonable approach in treatment of SAH.

Ursolic acid (UA), a natural pentacyclic triterpenoid acid, is one of the major components of certain medicinal plants. Many triterpenoids have been used for medicinal purposes for a variety of clinical diseases in many Asian countries [8]. Evidences have shown that UA possesses a wide range of biological effect, such as anti-oxidative biological effect, anti-apoptosis, anti-tumor, and anti-inflammatory activity [4,10,23-25]. It has been proven that UA could protect against oxidative stress- or neurotoxin-induced damage in vitro and in vivo [11,18,21]. The recent work by Li et al. has shown that UA protected the brain against ischemic injury by anti-oxidative and anti-inflammatory effect after middle cerebral artery occlusion in vivo [9]. Most recently, UA has been used for

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the treatment of malignant gliomas, which could induce tumor cell autophagy via reactive oxygen species (ROS)-dependent endoplasmic reticulum stress in vitro [17]. However, the effect of UA against SAH is still unknown. In this study, we investigated the potential effect of UA against SAH, and explored whether this effect is associated to its powerful antioxidative property.

## 2. Meterials and methods

### 2.1. Animal preparation and administration

The experimental protocol of using animals was conformed to the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. Male SD rats were purchased from Experimental Animal Central of General Hospital of Shenyang Military Command (Shenyang, China), fed on standard pellet chow and water ad libitum. Rats weighing between 250 and 300 g were randomly divided into four groups: (1) sham + vehicle group ($n = 18$), (2) SAH + vehicle group ($n = 18$), (3) SAH + 25 mg/kg UA group (SAH + UA25, $n = 18$), and (4) SAH + 50 mg/kg UA group (SAH + UA50, $n = 18$).

UA (Tianjin Shilan technology co., LTD, China, 98% pure) was intraperitoneally (i.p.) administered at 0.5, 24 and 47 h after SAH. An equal volume of Tween 80 served as the vehicle control. Forty-eight hours after SAH induction, one-third of the rats in each group were decapitated to obtain brain tissue samples for the biochemical analyses, the other one-third were decapitated for Evans blue (EB) assay and edema evaluation, and the remaining rats were given the fixative perfusion for histological preparation and analysis.

### 2.2. Experimental SAH rat model

The rat SAH model was performed by endovascular perforation as in previous study [2]. Briefly, rats were anesthetized with 1% sodium pentobarbital (35 mg/kg, i.p.). After exposing the left common carotid artery, external carotid artery (ECA) and internal carotid artery (ICA), the ECA was ligated, cut, and shaped into a 3-mm stump. A sharpened 4–0 monofilament nylon suture was advanced rostrally into the ICA from the ECA stump until resistance was felt (18–20 mm from the common carotid artery bifurcation) and then pushed 3 mm further to perforate the bifurcation of the anterior and middle cerebral arteries. After punctured 15 s, the suture was withdrawn into the ECA stump, and the ICA was reperfused to produce SAH. Sham-operated rats underwent the same procedure without perforation. The incision was then closed, and rats were housed individually following their recovery from anesthesia.

### 2.3. Neurological score and SAH severity evaluation

Neurological score was evaluated at 24 h after SAH according to the score system of Sugawara [20]. The severity of SAH was also quantified by Sugawara's grading scale [20] ($n = 6$), the basal cistern was divided into six segments, depending on the amount of subarachnoid blood clot to allotted a grade from 0 to 3.

### 2.4. Evaluation of the brain edema

After the rats were sacrificed, brains were quickly removed and weighted (wet weight). Brain specimens were dried in an oven at 110 °C for 72 h and weighted again (dry weight). The percentage of water was calculated according to the following formula: % $H_2O$ = [(wet weight-dry weight)]/wet weigh × 100.

### 2.5. Assay of blood–brain barrier disruption

Blood–brain barrier (BBB) permeability was quantitatively evaluated by EB extravasation at 48 h after SAH. EB dye (2%, 5 ml/kg) was injected into the left femoral vein and allowed to circulate for 1 h. Under deep anesthesia, rats were sacrificed by intracardial perfusion with saline and brains were removed. The entire brain of each was weighted, then immersed in formamide (10 ml/g) and incubated at 60 °C for 24 h. The extravasations were measured for absorbance of EB at 620 nm using a spectrophotometer.

### 2.6. The determination of lipid peroxidation and oxidative stress

The cerebral cortex was obtained and immediately frozen in liquid nitrogen until use ($n = 6$). The activity of Malondialdehyde (MDA), Glutathione (GSH), oxidized glutathione (GSSH) and Superoxide dismutase (SOD) was measured respectively by commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), the catalase activity assay kit was the product of Beyotime Co. (Haimen, China). These experiments were performed according to the manufacturer's instructions. All standards and samples were run in duplicate. The tissue protein concentration was determined using the BCA Protein Assay Kit.

### 2.7. Quantitative real-time polymerase chain reaction

Total RNA was extracted from brain tissue using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instruction and subjected to a quantitative real time polymerase chain reaction analysis. β-actin was served as an endogenous control. Rat-specific primers for caspase-3, caspase-9 were synthesized by AuGCT DNA-SYN Biotechnology Synthesis Lab (Beijing, China). Forward and reverse primers were 5′-ATGGACAACAACGAAACCTC-3′ and 5′-TTAGTGATAAAAGTACAGTTCTT-3′ for caspase-3, 5′-CTGAGCCAGATGCTGTCCCATA-3′ and 5′-CCAAGGTCTCGATGTACCAGGAA-3′ for caspase-9, 5′-CTGTGTGGATTGGTGGCTCTATC-3′ and 5′-TGTTATGATGGTGCCACTTGA-3′ for β-actin. Melting curve, which was measured immediately after amplification, showed single product peak, indicating good product specificity.

### 2.8. Western blot analysis

Brain samples from the ipsilateral basal cortex were lysated on ice for 30 min with RIPA lysis buffer and then centrifuged (12,000 g, 20 min, 4 °C). Protein concentration was determined using the BCA Protein Assay Kit. Western blot analysis was performed according to standard procedures. Rabbit anti-Caspase-3 polyclonal antibody (Boster, diluted 1:200) and Rabbit anti-Caspase-9 polyclonal antibody (Boster, diluted 1:200) were used as primary antibodies. Mouse β-actin monoclonal antibody (Boster, diluted 1:500) was served as a loading control.

The membranes were adequately washed with tris-buffered saline containing Tween 20 after each treatment with antibody. The membranes were developed with pro-light HRP Chemiluminescent kit (TIANGEN biotech, Beijing) and exposed to X-ray film. The protein levels of caspase-3 and -9 were expressed as the ratio of band optical intensity to β-actin.

### 2.9. Histopathologic detection

Rats ($n = 6$) were deeply anesthetized and sacrificed by intracardial perfusion with 4% ice-cold paraformaldehyde (pH 7.4). The formalin-fixed tissues were embedded in paraffin and sectioned at 4 μm thickness. Basilar artery sections were used to examine the cerebral vasospasm.