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# Therapeutic effects of Saussurea involucrata injection against severe acute pancreatitis- induced brain injury in rats☆

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ABSTRACT

*Objectives:* To observe the therapeutic effects of Saussurea involucrata (Sau) injection against severe acute pancreatitis (SAP)-induced brain injury.
*Methods:* Sodium taurocholate-induced SAP-modeled rats were equally randomized into an SAP model group (SAP group) and a Sau treated group (Sau + S group). Healthy rats were equally randomized into a Sau treated group (Sau + H group) and a sham operation group (SO group). Serum amylase levels, endothelin-1 (ET-1) and nitric oxide (NO) contents were determined by optical turbidimetry, ELISA and nitrate reductase method respectively. Western blot was used to detect protein expression levels of phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt), ET-1, inducible nitric oxide synthase (iNOS) and endothelial NOS (eNOS) while mRNA levels of these biomarkers in brain tissue were measured by quantitative real-time PCR. Furthermore, pathological changes, as well as all above indexes of pancreas and brain, were observed at 6, 24 and 48 h after administration.
*Results:* There was a significant difference in mortality between SAP and Sau + S groups ($P < 0.05$). Serum amylase levels, ET-1 and NO contents, ET-1/NO ratio, relative expression levels of ET-1 and iNOS protein/mRNA of brain tissue in Sau + S group were lower than those in SAP group at 24 and 48 h post-operation ($P < 0.05$ or 0.01), meanwhile, pancreas and brain pathological scores showed similar tendency ($P < 0.01$). However, both protein and mRNA levels of PI3K, Akt and eNOS of brain tissue in Sau + S group were higher than those in SAP group ($P < 0.05$ or $P < 0.01$). There were no significant differences in all indexes between Sau + H and SO groups at all designated time points ($P > 0.05$).
*Conclusions:* Sau injection has therapeutic effects on SAP-induced brain injury in rats.

## 1. Introduction

Severe acute pancreatitis (SAP) is a common clinical emergency characterized by the sudden occurrence and rapid progression. It is usually accompanied by systemic inflammatory response syndrome and multiple organ dysfunction syndromes (MODS), causing a 20–30% mortality and incurring heavy burdens on both families and society [1]. Brain injury, also known as pancreatic encephalopathy (PE), is one of the SAP-induced complications, involving 9–20% SAP patients and causing a mortality of 10.00-66.67% [2]. The main manifestations of PE include disorientation, confusion, hallucination and other abnormal mental states [3]. However, the pathogenic mechanisms in PE are not completely understood. Clinical treatment measures for PE are not effective enough partly due to its elusive pathogenesis. Currently, the role of microcirculatory disturbance in SAP has aroused increased attention and interest. Studies [4–6] have shown that microcirculatory disturbance is a trigger factor in the development of SAP and plays a key role in the development of MODS, which results in a high death rate.

Extracted from Saussurea involucrata (Sau) flowers, Sau injection has anti-inflammation and oxidation effect, moreover, it can also improve microcirculation, modulate immunity and protect the nervous system from injury, which mainly relies on total flavonoids, a group of main active constituents in this injection [7–14]. Studies [15–17] have demonstrated that Sau injection is safe and reliable for clinical use

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because it does not have significant toxicity and adverse effects. Our earlier research results [18] showed that Sau injection had a therapeutic effect against SAP in rats. In addition, other studies [19–21] have also shown that Sau injection is effective in treating various types of brain injury. Hereby we speculated that Sau injection should be effective for treating SAP-induced brain injury.

As there are few reports about the clinical use of Sau injection for the treatment of SAP-induced brain injury, we first established a rat SAP-induced brain injury model and then used it to observe the therapeutic effects of Sau injection on SAP-induced brain injury in present study. By using this model, we explored potential underlying mechanisms via multiple methods attempting to provide a solid experimental basis for clinical use of Sau injection in treatment of SAP-induced brain injury.

## 2. Materials and methods

### 2.1. Ethics declarations

This study was reviewed and approved by Danyang People's Hospital Institutional Review Board (No.: 2015-06-10). All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Nantong University School of Medicine (Nantong, China) in accordance with the Guide for the Care and Use of Laboratory Animals (Eighth Edition, 2011, published by the National Academies Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA).

### 2.2. Preparation of animals

Specific pathogen free (SPF)-grade healthy male Sprague-Dawley (SD) rats aged 10–12 weeks and weighing 250–280 g were supplied by and maintained in the Animal Experimental Center of Nantong University School of Medicine with a 12 h light/dark cycle and free access to standard laboratory feed and water.

### 2.3. Preparation of Sau injection

0.5% endotoxin-free Sau injection (containing 5 mg/ml total flavonoids) was purchased from Xinjiang Xiyu Pharmaceutical Co., Ltd (Urumqi, China; Approval No. Z65020023). Preparation protocol of Sau injection was provided by Xinjiang Xiyu Pharmaceutical Co., Ltd. as follows: Sau flower 1000 g was impregnated for 14 days, added with 85% ethanol, filtrated and concentrated to 1 ml solution containing 1 g raw herb. The solution was then sterilized in condition of 110°C 0.1kpa for 45 min, then tranquillized for 1–3 months and filtered. After addition of 804 ml polysorbate and 10 g sodium chloride, the filtrate was stirred intensively and adjusted pH to 6.0 ~ 8.0. Next, 40% sodium hydroxide solution was added, and then the mixture was further filtered through a 0.2 μm microporous membrane. At last, obtained filtrate was dissolved into 1000 ml injection with water, sealed and sterilized for use.

### 2.4. Experimental model and groups

All rats were fasted for 12 h with free access to water prior to operation. After anaesthesia with intraperitoneal (i.p.) injection of 2% sodium pentobarbital (0.25 ml/100g), they were laid and fixed on the table, routinely shaven, disinfected, and draped. For rat SAP model, freshly prepared 3.5% sodium taurocholate solution (Sigma-Aldrich, USA, 0.1 ml/100g) was retrogradely infused uniformly at the speed of 0.1 ml/min into biliopancreatic duct after laparotomy [22]. 3.5% sodium taurocholate solution was replaced by equivalent volume of normal saline solution in both sham-operation (SO) group and Sau + H group (healthy rats were treated with Sau injection only). The incision was closed with continuous 3-0-silk suture, and 2 ml/100g saline was injected into back subcutaneously (s.c.) to compensate for fluid loss. Acute toxicity test of Sau injection was performed as described in the literature [21]. According to drug use manual of Sau injection, the daily dosage for human use was 2 ml to 4 ml. The dose conversion coefficient between humans and rats was 12. In this study, the dose for rats was determined by considering conversion coefficient and contents of effective components in Sau injection. Briefly, the test was first trialled with 0.5% (5 mg/ml) Sau injection at a dose of 0.26 ml/kg to ensure safety, and then SAP modelled rats were treated with low, moderate and high doses of Sau injection. The result showed that high dose (1.04 ml/kg body weight) Sau injection can present optimal therapeutic effect without causing significant acute toxicity, therefore, this dosage was chosen for further study.

Totally 180 rats were equally randomized into four groups: (1) Sau injection treatment group (Sau + S group) in which the SAP model rats were injected i.p. with 0.5% Sau injection at a dose of 1.04 ml/kg; (2) SAP group in which the SAP model rats received an equivalent volume of normal saline (NS) via i.p.; (3) Sau + H group in which the healthy rats were injected i.p. with 0.5% Sau injection at a dose of 1.04 ml/kg; and (4) SO group in which the sham operation rats received an equivalent volume of NS via i.p.. All treatments were conducted after 6 h of successful modelling, and then administered every 12 h. 45 animals in each of the four groups were equally randomized into 6-, 24- and 48-h subgroups for postoperative observations. Surviving rats were sacrificed for taking blood and tissue samples at 6, 24 and 48 h in the four groups. The total sample size was too small in group of rats sacrificed at 6 h and 24 h. In order to observe death rate accurately, extra 60 rats were equally randomized to two groups: SAP group and Sau + S group. No rats were killed at halfway. These 60 rats were observed for 48 h.

### 2.5. Mortality

The rat mortality was calculated at 6, 24 and 48 h in all four groups.

### 2.6. Blood and tissue preparation

Surviving rats were sacrificed by taking blood via intracardiac puncture at 6, 24 and 48 h in four groups. Blood samples were collected for centrifugation, and serum was stored at −20°C. After sacrificing, a piece of fresh rat pancreas tissue and brain tissue were removed immediately and fixed in 4% phosphate-buffered formaldehyde for histopathologic observation. For transmission electron microscopy(TEM) observation, fresh removed brain tissue was cut into the size of 1 $m^3$ and fixed in 2% glutaraldehyde. The remaining brain tissues were immediately frozen in liquid nitrogen and then stored at −80°C for Western blot and quantitative real-time polymerase chain reaction (qRT-PCR) assay.

### 2.7. Histology

Pancreas tissue histological examination: hematoxylin-eosin (HE) staining was employed after specimens were fixed in formaldehyde, paraffin embedded, cut into 4 μm thick sections. In accordance to Schmidt's severity score standard of pancreas [23], edema, inflammation, glandular cell necrosis (each 0–4 points) and hemorrhage (0-1 point) of the pancreatic tissue were scored and recorded. For each pathological section, 10 visual fields under a high-power microscope (×200) were randomly selected and scored by two independent pathologists who were blind to this experiment. The mean score of the 10 visual fields of one pathological section was calculated as the pathological score.

Brain tissue histological examination: The brain tissue samples for HE were prepared and the examination method is the same as that for the pancreas tissue. For TEM observation, rat brain tissue specimens were fixed in 2% glutaraldehyde for 24 h, stained with acetic acid

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