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Original Article

Stragaloside IV weakens cognitive deficits of septic-associated encephalopathy through oxidative stress, induced nitric oxide synthase and inflammation in a mouse model

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Abstract: Stragaloside IV with immune regulation, organ protection, hypoglycemic, anti-inflammatory, antiviral, adjust the cell apoptosis and many pharmacological functions such as reproductive toxicity. We investigated for the first time whether the potential protective effect of stragaloside IV weakens cognitive deficits of septic-associated encephalopathy (SAE) in a mouse model. Moreover, the possible mechanisms of protection were also explored. Mice were received with a dose of 9 mg/kg stragaloside IV body weight for 20 weeks. Open Field Tests, Morris Water Maze Tests, Y Maze Tests and Serum Ammonia Assay were used to quantify the treatment with stragaloside IV on cognitive deficits in SAE rats. Microvessel density (MVD), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-PX), Nuclear transcription factor-kappa (NF-κB) p65 unit and Tumor necrosis factor alpha (TNF-α) were analyzed by ELISA kits. The inducible nitric oxide synthase (iNOS) protein expression was measured using western blotting analysis. Administration of stragaloside IV significantly weakens cognitive deficits, reduced the MDA, CAT, SOD, GSH-PX, NF-κB p65 unit and TNF-α levels, suppressed the iNOS protein expression in SAE rat. Our study indicated that stragaloside IV could prevent cognitive deficits of SAE rat through anti-oxidative and anti-inflammation.

Keywords: Stragaloside IV, septic-associated encephalopathy, oxidative, inflammation, iNOS

Introduction

Sepsis is the systemic inflammatory response syndrome caused by infection, its further development can lead to septic shock and multiple organ dysfunction syndrome [1]. Europe and the United States die because of sepsis for over 350000 each year, and treatment costs reach to $25 billion, it brings huge burden to the individual, family and the society [2]. Septic-associated encephalopathy (SAE) are one of higher morbidity and mortality disease of Sepsis complications, the scholars at home and abroad are committed to study SAE, and to find effective treatment measures [3]. In the clinical work, we need to know and judge the existence of SAE in time, and timely intervention, to prevent illness development, and reduce sepsis mortality [4].

The SAE refers to the professional activities or short or medium term exposure to a large number of chemicals is given priority to with nervous system damage caused by systemic diseases [5]. Some patients in recovery period will appear different degree of cognitive dysfunction. The SAE performance for long term autonomic nerve dysfunction, delirium and cognitive dysfunction, the exact mechanism is unclear, and the lack of effective treatment measures [6]. Studies have shown that mitochondrial dysfunction in Sepsis occurrence and development of multiple organ dysfunction plays an important role, speculated that mitochondrial dysfunction may be one of SAE pathophysiology mechanism [7].

Along with the advance of research techniques, the application of modern technology, found as

main active ingredients of astragalus Stragaloside IV, have to regulate the body's immunity, protect tissues and organs, reduce blood sugar, apoptosis and anti-inflammatory antiviral resistance etc many pharmacological functions [8]. At the same time, we should pay attention that stragaloside IV has certain reproductive toxicity, so pregnant women should avoid using it [9]. From the current research results show that stragaloside IV is a good potential new drug development [10].

Therefore, we hypothesized that the potential protective effect of stragaloside IV weakens cognitive deficits of septic-associated encephalopathy in mouse. Furthermore, the possible protection mechanisms of stragaloside IV on cognitive deficits were also explored in the present study. We used a mouse model of SAE and our study indicated that stragaloside IV weakens cognitive deficits of SAE through oxidative stress, induced nitric oxide synthase and inflammation in a mouse model.

Materials and methods

Animals and agents

Health adult male C57BL/6 mice (26-32 g) was obtained from experiment center of Wuhan University, and maintained under a 12 h dark/light cycle (23-24°C, relative humidity 40-60%). After starting experiment, all mice were fed a standard labomiceory diet and water ad libitum in the labomiceory for 1 week. All studies performed on animals were approved by the Institutional Animal Care and Use Committee. Sodium pentobarbital was obtained from Sigma Chemical Co, St. (Louis, MO, USA). Microvessel density (MVD), Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-PX), Nuclear transcription factor-kappa (NF-κB) p65 unit and Tumor necrosis factor alpha (TNF-α) ELISA kits were obtained from Jiancheng Bioengineering Institute (Nanjing, China).

Model of septic encephalopathy

All mice were anesthetized with 40 mg/kg of sodium pentobarbital (2% saline, intraperitoneally, ii, Sigma Chemical Co, St. Louis, MO, USA). Then, under anesthesia, cecum was isolated and ligated using 4.0 silk. A sterile 22-gauge needle was used to puncture the cecum twice on the anti-mesenteric side. The cecum was gently squeezed to extrude the fecal contents into the peritoneal cavity, placed back into the abdomen and closed with sutures in two layers. Some model mice were exposed in the same manner as cecal ligation and puncture. A mouse model of SAE was induced by cecal ligation and puncture.

Experimental grouping

Normal mice (n = 10) were received with a delayed administrate of saline, which was deed as control group. Normal mice (n = 10) were received with a dose of 9 mg/kg stragaloside IV body weight for 20 weeks, which was deed as IV group. SAE mice were (n = 10) were received with a delayed administrate of saline, which was deed as SAE group. SAE mice were (n = 10) were received with a dose of 9 mg/kg stragaloside IV body weight for 20 weeks, which was deed as SAE IV group. At 20 weeks after operation, the survived mice were subjected to behavioral tests and tissue analysis.

Open field tests

Open Field Tests were tested to determine anxiety-related behavior. The open field was putted into 9 equal rectangles and then mice were placed in the center quadrant. The same plastic chamber (40 × 40 × 45 cm) was used to test Habituation for 5 min. In the center and borders of the open field, the total distance traveled was recorded. After each mouse was conducting a test, the apparatus was cleaned with 70% ethanol.

Morris water maze tests

Morris Water Maze Tests were tested to determine the spatial learning and memory function. The diameter of 120 cm of water maze pool contained opaque water. The diameter of 10 cm platform was placed in one quadrant of the pool. All mice were given four trials per day to locate the hidden platform for spatial training sessions over 4 consecutive days. Then, the platform was removed at 4 h after the last training session and a 60 s probe trial was performed.

Y maze tests

Y Maze Tests were tested to determine the aversive memory of mice as previously described [11]. All mice were not subjected to further laboratory analysis.

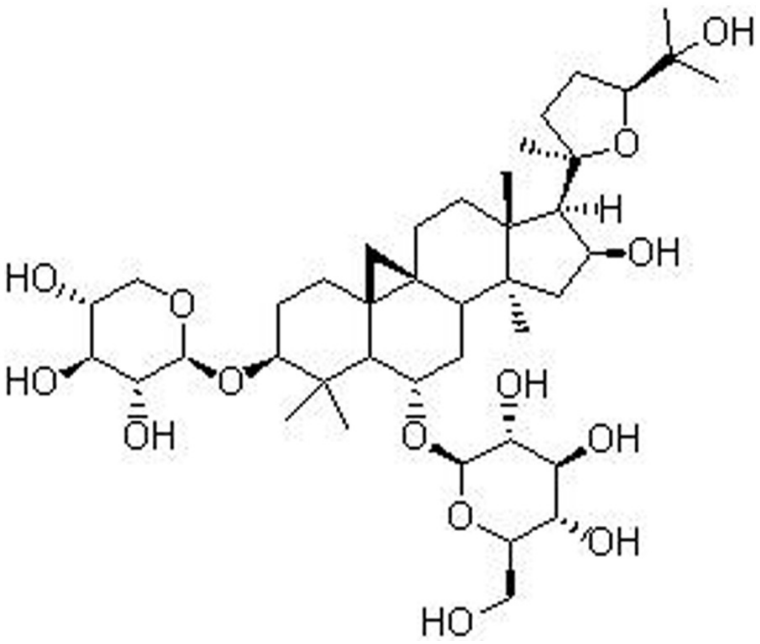

Figure 1. The chemical structure of stragaloside IV.

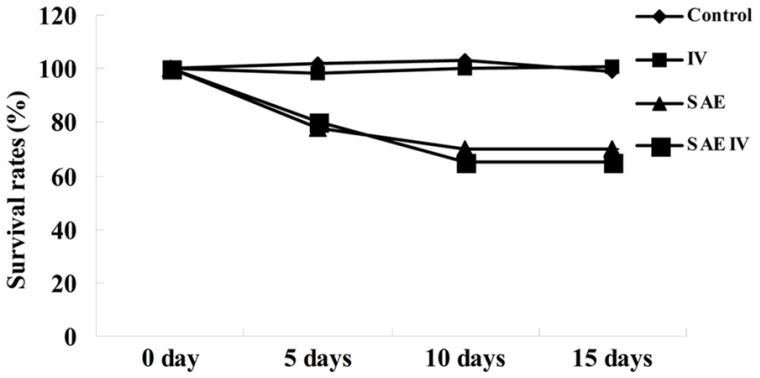

Figure 2. Survival rates.

Serum ammonia assay

Under anesthesia, a midline thoracotomy was performed and the left ventricle was inserted with 20-gauge needle to collect blood sample. The blood sample was centrifuged at 3,000 rpm for 10 minutes and the serum ammonia levels were determined by an autoanalyzer (Hitachi 7060, Tokyo, Japan).

Detections of MDA, CAT, SOD and GSH-PX

The blood sample was centrifuged at 3,000 rpm for 10 minutes and the serum MDA, CAT, SOD and GSH-PX levels were determined by the ELISA kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturers' instructions.

Detections of NF-κB p65 unit and TNF-α

The blood sample was centrifuged at 3,000 rpm for 10 minutes and the serum NF-κB p65 unit and TNF-α levels were determined by the thiobarbi turic acid method (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturers' instructions.

Western blotting analysis of inducible nitric oxide synthase (iNOS)

All mice were euthanized with an overdose of sodium pentobarbital. Brain tissue sample was homogenated using liquid nitrogen and added with RIPA lysis buffer (Beyotime Inc, Jiangsu, China) and centrifuged at 3,000 rpm for 10 minutes. Then, supernatant was collected to quantify the protein contents with Coomassie brilliant blue assay (Beyotime Inc, Jiangsu, China). Equivalent protein was separated by electrophoresis using 12% SDS-polyacrylamide gels. After being transferred to nitrocellulose filter membranes, the membranes were blocked in 5% skimmed milk and incubated with the corresponding primary antibodies anti-iNOS (1:1000, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and β-actin (1:5000, Santa Cruz Biotechnology Inc., Santa Cruz, CA) overnight at 4°C. Then, the membranes were washed with TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween-20) and incubated with secondary antibodies (1:5000, BestBio Inc, Shanghai, China) for 2-3 h °C and appended with an enhanced chemiluminescence detection (ECL, Amersham, Arlington Heights, IL).

Statistical analysis

Data are expressed as mean ± S.E.M. and performed using an unpaired Student's t test. Comparisons among multiple groups involved one-way ANOVA followed by Tukey multiple

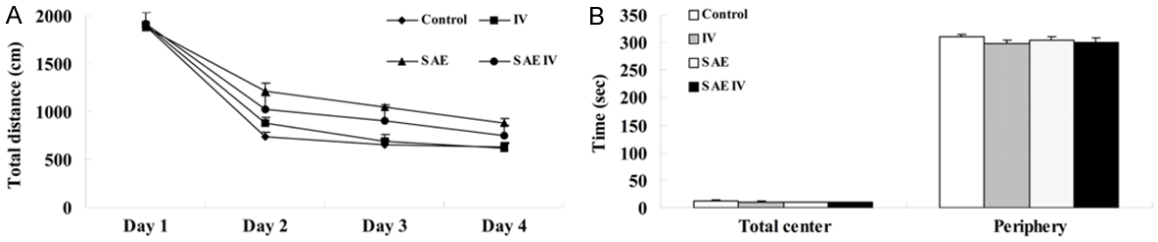

Figure 3. Habituation to the open field. The total distance (A), the center or the periphery of the time spent (B). Control, Control group; IV, Stragaloside IV group; SAE, septic-associated encephalopathy group; SAE IV, septic-associated encephalopathy + Stragaloside IV group. $^{**}P < 0.01$ compared with Control group; $^{\#\#}P < 0.01$ compared with SAE group.

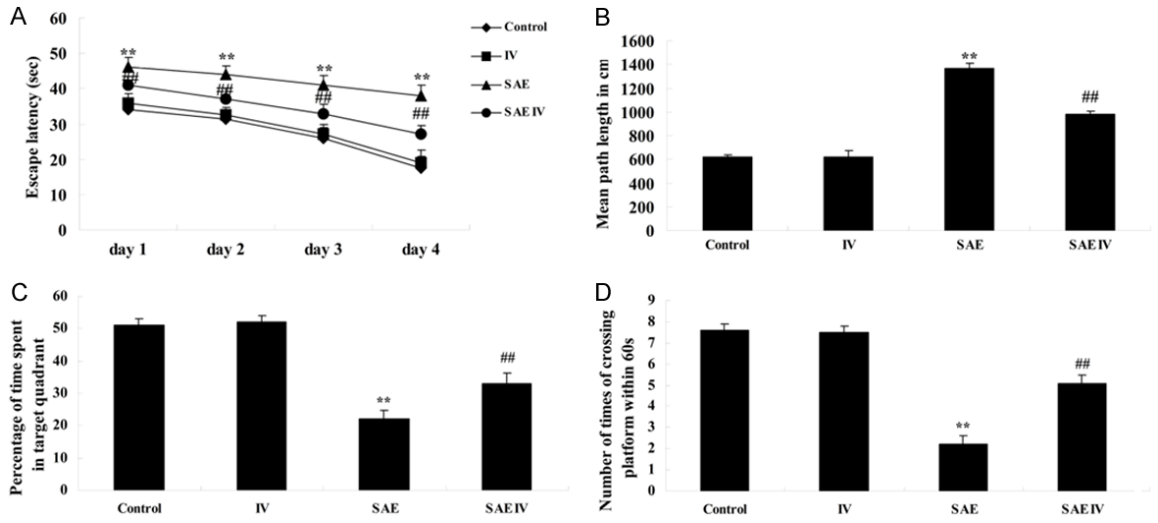

Figure 4. Performance on Morris water maze. The escape latency (A), mean path length (B), mean swim speed (C) and spent more time (D). Control, Control group; IV, Stragaloside IV group; SAE, septic-associated encephalopathy group; SAE IV, septic-associated encephalopathy + Stragaloside IV group. $^{**}P < 0.01$ compared with Control group; $^{\#\#}P < 0.01$ compared with SAE group.

comparison testing with $P < 0.05$ considered statistically significant.

Results

Survival rates

The structure chart of stragaloside IV (Sigma-Aldrich Co. LLC, Germany, purity with 98%) demonstrated **Figure 1**. In the present study, the survival rate was recorded. In the first 10 days, the death rate of rat was mainly distributed after SAE. In control group and IV group, no difference of the death rate was surveyed and zero rat death was appeared after SAE (**Figure 2**). When stragaloside IV was administrated 15 days, there were no additional rat deaths after the initiation of VPA treatment (**Figure 2**).

Habituation to the open field

Open Field Tests was used to analyze the protective effect of stragaloside IV on habituation in SAE rats. In the total distance, the center or the periphery of the time spent, there were significant differences between all the experiments (**Figure 3**).

Performance on Morris water maze

Morris Water Maze Tests was used to analyze the protective effect of stragaloside IV on performance in SAE rats. In the training sessions, escape latency of control rat was similar to that of IV group (**Figure 4A**). SAE observably induced the escape latency after CLP, in comparison to that of control group (**Figure 4A**). The advance escape latency was observably reduced by stragaloside IV treatment in SAE rat (**Figure**

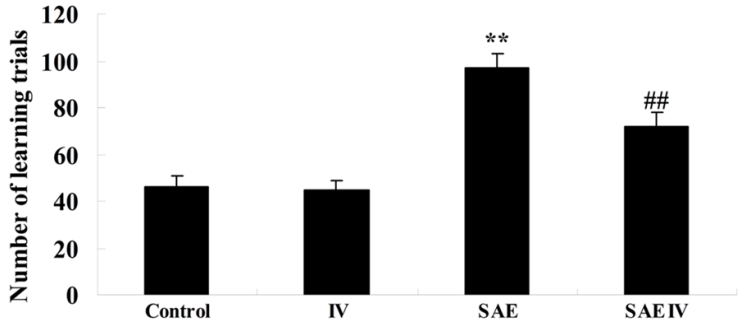

Figure 5. Number of learning trials in the Y maze. Control, Control group; IV, Stragaloside IV group; SAE, septic-associated encephalopathy group; SAE IV, septic-associated encephalopathy + Stragaloside IV group. $^{**}P < 0.01$ compared with Control group; $^{\#\#}P < 0.01$ compared with SAE group.

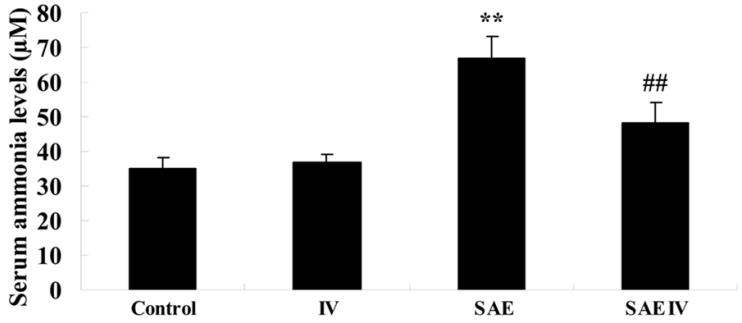

Figure 6. Changes of serum ammonia concentrations. Control, Control group; IV, Stragaloside IV group; SAE, septic-associated encephalopathy group; SAE IV, septic-associated encephalopathy + Stragaloside IV group. $^{**}P < 0.01$ compared with Control group; $^{\#\#}P < 0.01$ compared with SAE group.

4A). In path length, there was no significant difference between control group and IV group (**Figure 4B**). SAE observably increased the mean path compared to that of control group (**Figure 4B**). The elevation mean path was significantly inhibited by treatment with stragaloside IV after CLP in rat (**Figure 4B**).

We found no significant inter-group difference between control group and IV group for the mean swim speed and spent more time in SAE rat (**Figure 4C**, **4D**). As compared to that of control group, the mean swim speed and spent more time were significantly weakened in SAE rat (**Figure 4C**, **4D**). When pretreatment with stragaloside IV, the mean swim speed and spent more time was significantly suppressed in comparison to that of SAE group (**Figure 4C**, **4D**).

Number of learning trials in the Y maze

Y Maze Tests was used to analyze the protective effect of stragaloside IV on the number of learning trials. However, in the number of learning trials, no significant changes amongst between control group and IV group were observed (**Figure 5**). Our results showed that the number of learning trials in SAE group was markedly induced after CLP compared to that of control group (**Figure 5**). Administrate of stragaloside IV markedly decreased the number of learning trials in SAE rat (**Figure 5**).

Changes of serum ammonia concentrations

We determined that the protective effect of stragaloside IV on the serum ammonia concentrations in our study. The serum ammonia level of control group was very similar to IV group (**Figure 6**). As shown in **Figure 6**, SAE significantly increased the serum ammonia level in SAE (**Figure 6**). However, pretreatment with stragaloside IV significantly reduced the serum ammonia level in SAE rat compared to that of SAE group (**Figure 6**).

Changes of MDA, CAT, SOD and GSH-PX

To determine the effect of stragaloside IV on SAE, the serum MDA, CAT, SOD and GSH-PX levels were determined by the ELISA kit. As shown in **Figure 7**, no difference of the serum MDA, CAT, SOD and GSH-PX levels was appeared after SAE in control group and IV group. However, SAE effectively promoted the serum MDA level and suppressed the serum CAT, SOD and GSH-PX levels in rats (**Figure 7**). Interesting, the tendencies were effectively reversed by treatment with stragaloside IV compared to that of SAE group (**Figure 7**).

Changes of NF-κB p65 unit and TNF-α

To measure the effect of stragaloside IV on SAE, the serum NF-κB p65 unit and TNF-α levels were determined by the ELISA kit. However, the serum NF-κB p65 unit and TNF-α levels, no significant changes amongst between control group and IV group were seen (**Figure 8**). The

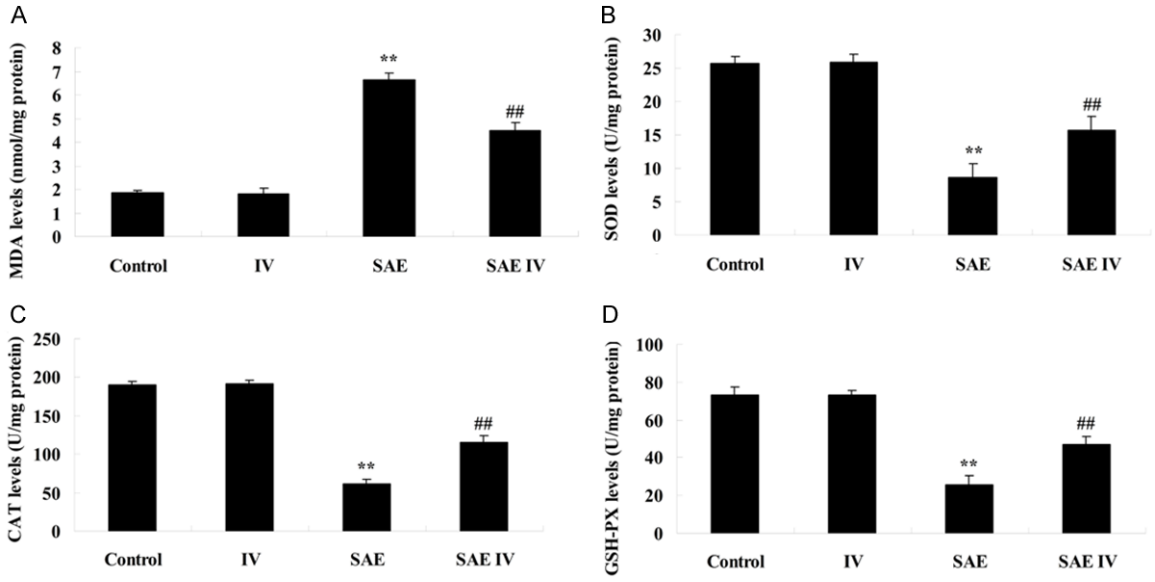

Figure 7. Changes of MDA, CAT, SOD and GSH-PX. Changes of MDA (A), CAT (B), SOD (C) and GSH-PX (D). Control, Control group; IV, Stragaloside IV group; SAE, septic-associated encephalopathy group; SAE IV, septic-associated encephalopathy + Stragaloside IV group. $^{**}P < 0.01$ compared with Control group; $^{\#\#}P < 0.01$ compared with SAE group.

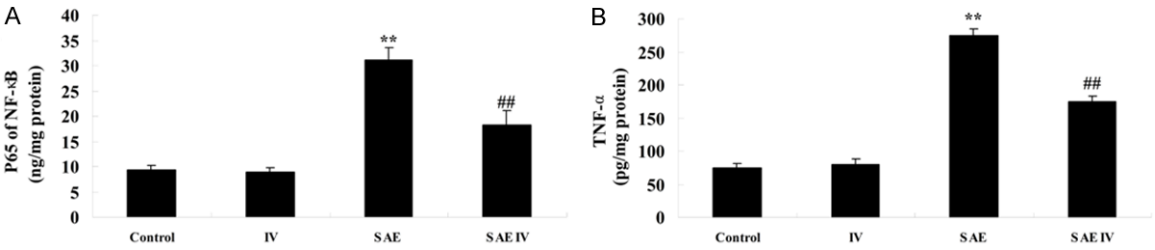

Figure 8. Changes of NF-κB p65 unit and TNF-α. Changes of NF-κB p65 unit (A) and TNF-α (B). Control, Control group; IV, Stragaloside IV group; SAE, septic-associated encephalopathy group; SAE IV, septic-associated encephalopathy + Stragaloside IV group. $^{**}P < 0.01$ compared with Control group; $^{\#\#}P < 0.01$ compared with SAE group.

serum NF-κB p65 unit and TNF-α levels in SAE rats were significantly enhanced compared to that of control group (**Figure 8**). However, heighten the serum NF-κB p65 unit and TNF-α levels were significantly inhibited by treatment with stragaloside IV compared to that of SAE group (**Figure 8**).

Changes of iNOS protein expression

To explore the effect of stragaloside IV on SAE, the iNOS protein expression was surveyed using western blotting analysis. Meanwhile, there was no significant difference between control group and IV group (**Figure 9**). SAE significantly increased the iNOS protein expression in rats compared to that of control group (**Figure 9**). However, the increase in the iNOS protein expression was significantly abolished by treatment with stragaloside IV compared to that of SAE group (**Figure 9**).

Discussion

As early as 2500 years ago by Hippocrates for the first time pointed out that the development of an abscess was discovered for cases of mental disorder, bright puts forward the concept of SAE for the first time in 1827 [12]. Sepsis in recent years advanced studies, but the SAE research and report is relatively small, its pathological physiology, pathogenesis, diagnosis and treatment is still controversy [13]. Sepsis patients with vascular endothelial cell damage, increasing capillary permeability, microcirculation dysfunction leads to ischemia,

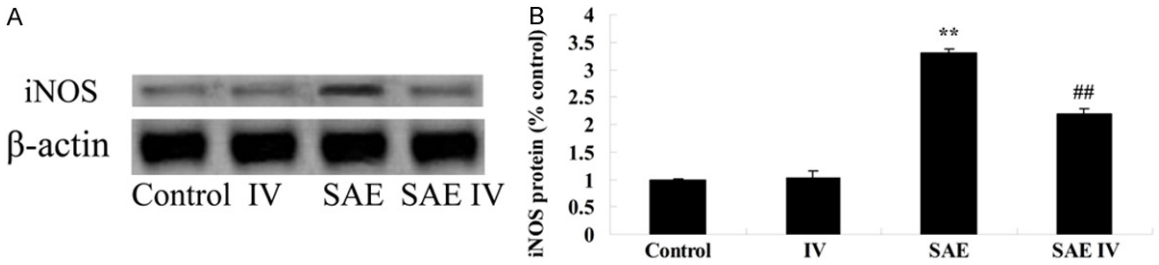

Figure 9. Changes of iNOS protein expression. Indicated representative western blotting analysis of changes of iNOS protein expression, and statistical analysis of iNOS protein expression. Control, Control group; IV, Stragaloside IV group; SAE, septic-associated encephalopathy group; SAE IV, septic-associated encephalopathy + Stragaloside IV group. $^{**}P < 0.01$ compared with Control group; $^{\#\#}P < 0.01$ compared with SAE group.

hypoxia and organ damage, the central nervous system is the most sensitive organ of ischemia and hypoxia [14]. Literature reported incidence of sepsis patients with SAE is 8%~70% [15]. Age, smoking history for a long time, coronary heart disease, diabetes, blood coagulation dysfunction, electrolyte disorder, APACHE II scoring ≥ 20 points more prone in sepsis patients with SAE. Diabetes, blood coagulation dysfunction and APACHE II ≥20 points are independent for risk factors of SAE. We found that the potential protective effect of stragaloside IV could weakens cognitive deficits, the advance escape latency, the elevation mean path, increased the drop of mean swim speed and spent more time, and inhibited the rise of the number of learning trials and the serum ammonia level in SAE rat. Kim et al. indicated that the effect of astragaloside IV ameliorates learning and memory deficit of rat with chronic cerebral hypoperfusion [16]. These data showed that the potential protective effect of stragaloside IV might be enhancing cognitive further in SAE rat.

After oxidative stress imbalances can through the following several mechanisms to produce cytotoxic effect: Direct damage mitochondria membrane structure; Change of enzyme activity; Affect the gene expression and transcription of genetic information; The mechanism of inducing cell apoptosis and oxidative stress induced cell apoptosis as follows: Oxidative stress status → mitochondrial membrane permeability changes → Cyt C into the cytoplasm in the mitochondria → activate the interleukin 1β converting enzyme family protease → induce apoptosis [17]. The some study found that in septic-associated encephalopathy patients, neutrophil oxidative stress and phagocytosis will enhance, produce a large number of reactive oxygen species, but also increase their apoptosis [18]. Oxidative stress is one of the important mechanisms of sepsis encephalopathy occurred, it will cause outbreaks of oxygen free radical, cause tissue damage, thus reducing oxidative stress reaction and their after products such as reactive oxide species, reactive nitrogen species, it is likely to be the treatment have certain effect to sepsis encephalopathy [19]. Studies have proved that the method of endotoxin induced sepsis heat shock preconditioning in rats, heat shock can cut down the iNOS, reduce the production of products after oxidative stress, so as to reduce the severity of the Sepsis encephalopathy [20]. In our study, astragaloside IV significantly significantly suppressed the oxidative stress and the iNOS protein expression in SAE rat. He et al. suggested that astragaloside IV inhibits oxidative stress in H9c2 cardiac cells [21]. Qiu et al. reported that astragaloside IV inhibits oxidative stress and ameliorates homocysteine-induced acute phase endothelial dysfunction [22]. He et al. suggested that astragaloside IV attenuates experimental autoimmune encephalomyelitis through suppression of iNOS expression in mice [23]. These results from our study, the anti-oxidative effect of stragaloside IV may be relevant to the therapy for SAE.

Inflammatory mediators out of control release is the root cause of sepsis morbidity and the disease progression, some organs such as brain, through the vagus nerve and ventricle around organs two pathways, the activation signal such as the before inflammation perception factors, induce inflammatory transmitter in synthesis and release of inflammatory medium adjustment [24]. A variety of inflammatory mediators in the brain can affect brain cells

through multiple ways after the increase of normal physiological and immune function, causing lack of oxygen to brain ischemia [25]. Studies suggest that inflammatory factors TNF-α direct damage mitochondrial membrane, show the mitochondrial transmembrane potential changes significantly reduced and the efficiency of oxidative phosphorylation and the expression of cytochrome decrease, brain use oxygen ability decline, aggravating anoxic brain damage [26]. In present study, astragaloside IV significantly inhibited the serum NF-κB p65 unit and TNF-α levels in SAE rats. Li et al. proposed that astragaloside IV suppressed the production of TNF-α and IL-1β and protects against focal cerebral ischemia/reperfusion injury [27]. Gui et al. suggested that astragaloside IV suppresses the renal NF-κB activity and TNF-α and ameliorates renal injury [28]. These results of our study implied that stragaloside IV, a novel anti-inflammatory agent, might be enhancing cognitive further in SAE rat.

In conclusion, our data demonstrated that neuroprotective, the anti-anti-oxidative and the anti-inflammatory function of astragaloside IV on SAE. Therefore, we propose that astragaloside IV may be a potential therapeutic approach for the treatment on SAE. Further clinical trials are needed for investigating the clinical efficacy of astragaloside IV.

Disclosure of conflict of interest

None.

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