doi.org/10.36721/PJPS.2020.33.6.REG.2567-2577.1

Alleviation of sepsis-associated encephalopathy by ginsenoside via inhibition of oxidative stress and cell apoptosis: An experimental study

Xiping Mei, Huibin Feng and Bibo Shao*

Department of Critical Care Medicine, Hubei Provincial Key Laboratory of Kidney Disease Occurrence and Intervention in Huangshi Central Hospital of Hubei Medical College (Affiliated Hospital of Hubei Institute of Technology), Huangshi, Hubei, China

Abstract: Ginsenoside (Rg1) has biological effects including anti-oxidation, anti-inflammation, neuroprotection and neural function improvement, but with few studies in sepsis-associated encephalopathy (SAE). This study thus evaluated Ginsenoside in alleviating SAE, suppressing oxidative stress (OS) or neuronal apoptosis. SAE mouse model was generated and were assigned into SAE, SAE + LD-Rg1, and SAE + HD-Rg1 groups to measure neural apoptosis by flow cytometry. Contents of malondialdehyde (MDA), superoxide dismutase (SOD), GSH-Px and caspase-3 were quantified, and mouse neural reflex function was evaluated. Expression of Nrf2, HO-1 was measured. Mouse neuron MN-c and microglia BV2 were co-cultured in control, LPS, LPS+Rg1 (20μM) and LPS+Rg1 (40μM) groups. Iba-1 expression of BV2 cells was measured by flow cytometry. Contents of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 were quantified. Apoptosis of MN-c cells was measured by flow cytometry, and reactive oxygen species (ROS) content was measured by DCFH-DA staining. SAE mice had elevated caspase-3 activity, cell apoptosis, MDA content, and decreased SOD, GSH-Px activity or neural reflex score comparing to Sham group. Rg1 treatment suppressed caspase-3 activity, apoptotic rate or MDA content, recovered SOD activity, neural reflex score, and expression of Nrf2 and HO-1. LPS treatment elevated Iba-1 expression and release of inflammatory cytokines TNF-α, IL-1β and IL-6, induced MN-c apoptosis or ROS production, and enhanced Nrf2 and HO-1 expression. Rg1 treatment remarkably inhibited LPS-induced response or cell apoptosis. Ginsenoside can alleviate SAE damage via up-regulating Nrf2 and HO-1 to enhance anti-OS potency and to reduce neural cell apoptosis.

Keywords: Sepsis-associated encephalopathy (SAE), ginsenoside, Nrf2, oxidative stress, cell apoptosis.

INTRODUCTION

Sepsis is one systemic inflammatory response syndrome (SIRS) caused by various pathogen infection and subsequently released endotoxins, and is one of severe complications in critical patients of trauma or shock (Caserta *et al.*, 2016; Xu *et al.*, 2018; Zrzavy *et al.*, 2018). Sepsis-associated encephalopathy (SAE) is one diffused brain dysfunction caused by systemic inflammation reaction and lacking clinical or laboratory evidences indicating central nervous infection (Feng *et al.*, 2017; Molnar *et al.*, 2018; Yamaga *et al.*, 2017). Clinical symptoms of SAE include delirium, coma, seizure episode or focal neurological manifestations (Chaudhry and Duggal, 2014). SAE is one of most common brain disorders in the intensive care unit (ICU). Patients complicated with SAE had remarkably elevated mortality rate reaching up to 40%~50% (Cotena and Piazza, 2012; Zhang *et al.*, 2012). SAE has extremely complicated pathogenesis mechanisms which have not been fully illustrated. Current ideas believe that dysfunction of blood brain barrier (BBB) or cerebral micro vascular circulation, inflammatory mediator release, abnormality of amino acid or neurotransmitter, mitochondrial dysfunction, oxidative stress (OS) and neuronal apoptosis all may participate in the onset and progression of SAE (Ji *et al.*, 2018; Kawakami *et al.*, 2018; Kuperberg and Wadgaonkar, 2017; Molnar *et al.*, 2018; Wu *et al.*, 2015).

**Corresponding author:* e-mail: nxv1tp9j9n@sina.com

Ginsenoside is one type of steroid compound and mainly exists in ginseng and has multiple pharmaceutical activities including anti-oxidation, anti-aging, anti-inflammation, decreasing cell apoptosis and protecting neurons (Jin *et al.*, 2017; Li *et al.*, 2016; Wang *et al.*, 2018). Various studies have shown the protective role of Ginsenoside in multiple brain disorders such as Parkinson's disease (Jiang *et al.*, 2015), Alzheimer's disease (Li *et al.*, 2016) and cerebral ischemia (Yang *et al.*, 2015). Ginsenoside has been shown to play important protective roles in sepsis, including anti-inflammation, anti-oxidation and maintenance of mitochondrial function (Xing *et al.*, 2017; Zou *et al.*, 2013). However, few studies have been performed regarding the role of Ginsenoside in SAE. This study thus investigated the function of Ginsenoside in alleviating SAE brain damage, OS injury and brain cell apoptosis.

MATERIALS AND METHODS

Major reagent and materials

Mouse microglia cell line BV2 and cortical neuron cell line MN-c were purchased from Yubo Biotech (China). DMEM medium and fetal bovine serum (FBS) was purchased from Gibco (US). Ginsenoside Rg1, DCFH-DA, lipopolysaccharide (LPS) were purchased from Sigma (US). Trizol reagent was purchased from Invitrogen (US). Rabbit anti-mouse Nrf2, HO-1 and β-actin polyclonal antibody were purchased from Abcam

(US). HRP conjugated goat anti-rabbit secondary antibody was purchased from Sangon Bio (China). PrimerScript™ RT reagent Kit was purchased from Takara (China). Lipid per oxidation product malondialdehyde (MDA), super oxide dismutase (SOD), and glutathione peroxidase (GSH-Px) test kits were purchased from Jiancheng Biotech (China). Annexin V-FITC/PI cell apoptosis test kit, BeyoECL plus RIPA lysis buffer, BCA protein quantification kit were purchased from Beyotime (China). Tran swell chamber was purchased from Corning (US). PE labelled Iba-1 antibody was purchased from Biolegend (US). HERA cell 240i cell incubator was purchased from Thermo (US). Gallios flow cytometry was purchased from Beckman Coulter (US). CFX96 real-time fluorescent quantitative PCR was purchased from Bio-Rad (US).

Study of animals

Healthy male adult C57BL/6 mice (6~8 weeks, body weight 230~260g) were purchased from Hubei Provincial Key Laboratory of Kidney Disease Occurrence (Huangshi, Hubei, China). Mice were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Hubei Provincial Key Laboratory of Kidney Disease Occurrence (Huangshi, Hubei, China) (ethnic number: 20170609E).

Generation of SAE model

After 1-week acclimation feeding, C57BL/6 mice were prepared for SAE model using cecal ligation and puncture (CLP) approach. In brief, mice were fasted for 12h before surgery, and were anesthetized by sevoflurane inhalation. A middle incision was made on the abdominal region after sterilization to dissect subcutaneous tissues and peritoneal tissues. The cecum was freed and ligated at one half of the blind end using the suture silk and was punctured by 20G needles. After squeezing trace amounts of feces from the puncture site, the colon was returned, and the skin incision was closed. Subcutaneous perfusion of saline (3mL/100g) was applied for rehydration after surgery. Sham group received the surgery for abdominal cavity exposure only, without cecal puncture or ligation. Mice were given food and water *as libitum* after resuscitation.

24h after surgery, mice were evaluated for neural reflex function, and were anesthetized by intraperitoneal injection of 10% hydrate chloral. Saline was perfused into the aorta and brain tissues were collected for homogenization. Cell suspensions were filtered to prepare single-cell suspension. After two times of PBS rinsing by centrifugation, flow cytometry was performed to detect the apoptotic rate. Peripheral blood samples were collected to measure S100β content by ELISA.

Partial brain tissues were collected and homogenized for 1000g centrifugation for 20min. The supernatant from homogenate was saved and was quantified for measuring MDA, SOD and GSH-Px content.

Neural reflex evaluation

24h after surgery, mice were evaluated for neural reflex function before sacrifice and sample collection. In brief, pinnate reflex and corneal reflex were used to evaluate simple non-positional locomotor function, and tail reflex was used to evaluate simple positional function, and righting and escaping reflex were used to evaluate complex positional motor function. In pinnate reflex, the head-shaking response of mice was evaluated after gentle touching on external auditory meatus. Corneal reflex was performed by gentle touch of the corneal surface by cotton swab for consequent head-shaking. Tail and escaping reflex were evaluated for the occurrence of escape for negative stimuli by rapid tail squeeze and needle puncture. Righting reflex was evaluated by the time of mice in supine position for body rotating. For each reflex, a scale of 0-10 was given with 0 standing for non-response, 1 for minor reflex, and 2 for normal response.

Ginsenoside Rg1 treatment for SAE model mice

SAE model mice were randomly assigned into three groups: SAE model group which received equal volume of saline via gavage at 3 days before surgery. SAE + LD-Rg1 group received 15mg/kg Rg1 (dissolved in 0.5% carboxymethylcellulose sodium) daily for 3 days before surgery. SAE + HD-Rg1 group received 30mg/kg Rg1 by gavage daily for 3 days before SAE surgery.

24h after surgery, mice were evaluated for neural reflex function, and were collected for brain tissues for homogenization. Cell suspensions were filtered to prepare single-cell suspension. Reactive oxygen species (ROS) and cell apoptosis were measured by flow cytometry.

Partial brain tissues were collected and homogenized for 1000g centrifugation for 20min. The supernatant from homogenate was saved and was quantified for measuring MDA, SOD and GSH-Px content. Peripheral blood samples were collected to measure S100β content by ELISA.

Caspase-3 enzyme activity assay

Following the instruction of test kit, pNA standard samples at serial dilutions were prepared to measure absorbance (A) values at 405nm wavelength (A405). A standard curve was plotted based on A405 and pNA concentration. 100μL Caspase Lysis Buffer was added into each 3mg brain tissues and was homogenized on ice. The homogenate was transferred into the new tube for 5min lysis on ice, and was centrifuged at 10000g for 20min at 4°C. The supernatant of lysate was saved for measuring caspase-3 activity. 65μL Assay buffer, 25μL lysate supernatant, and 10μL Ac-DEVD-pNA were added

into 96-well plate for 37°C incubation for 2h. A405 was immediately measured. The relative enzymatic unit was calculated by using following formula: A405 of experimental group / A405 of control group X 100%.

Co-culture of BV2 and MN-c cells and grouping

MN-c cells were re-suspended into DMEM-F12 medium containing 10% FBS and were inoculated into 24-well plate. BV2 cells were inoculated into the upper chamber/ after 24h incubation in 37°C chamber with 5% CO_2, cells were treated with IL-1β and Rg1. In specific, control group received no special treatment. In LPS group, 2μg/mL LPS were added into the upper chamber. LPS + Rg1 (20μM) group received 2μg/mL LPS in the upper chamber and 25μM Rg1 in both upper and lower chambers. In LPS + Rg1 (40μM) group, 2μg/mL LPS was added into the upper chamber, and 50μM Rg1 was added into upper and lower chamber. After 48h continuous chamber, the culture medium from upper chamber was collected for measuring the content of inflammatory factors TNF-α, IL-1β and IL-6. BV2 cells were collected from the upper chamber for measuring the expression of microglia activation marker Iba-1. Cells from the lower chamber were collected to measure apoptosis and ROS content by flow cytometry.

Flow cytometry to measure intracellular ROS

MN-c cells from lower chamber of all treatment groups were digested by trypsin and were collected, followed by centrifugation and rinsing in PBS. Serum-free DMEM-F12 medium was used to dilute DCFH-DA probe at 0.1% concentration, followed by 37°C incubation for 20min, during which 3~5 times of gentle mixtures were performed between cells and ROS probes. Cells were then rinsed and centrifuged in serum-free DMEM medium for 3 times and were measured on flow cytometry immediately.

Flow cytometry for measuring cell apoptosis

MN-c cells were collected in the lower chamber from all treatment groups by trypsin and were rinsed in PBS by centrifuge. 100μL Binding Buffer was used to re-suspend cells, which were mixed with 5μL Annexin V-FITC for 15min dark incubation at room temperature. 5μL PI staining solution was added, followed by 400μL Binding Buffer. Cell apoptosis was measured by flow cytometry.

Flow cytometry for measuring the expression of microglia activation marker Iba-1

BV2 cells in the upper chamber from all treatment groups were digested by trypsin and were collected. After PBS rinsing in centrifugation, cells were fixed in 80% methanol, and were permeabilized by 0.1% PBS-Tween. After 20min blocking in PBS containing 10% goat anti-serum, PE labelled Iba1 primary antibody was added for 4°C incubation for 30min. After two times of PBS rinsing, positive rate of Iba1 expression was measured by flow cytometry.

qRT-PCR for measuring gene expression

Total RNA was extracted by Trizol, and cDNA was synthesized from RNA by reverse transcription using PrimeScript™ RT reagent Kit. Using cDNA as the template, PCR amplification was performed under TaqDNA polymerase. In a 10μL reaction system, one added 5.0μL 2XSYBR Green Mixture, 0.5μL forward and reverse primer (5μm/L), 1μL cDNA, and ddH_2O for topping to 10.0μL. Reverse transcription was performed under the following conditions: 50°C for 15min, followed by 85°C for 5min. PCR conditions were: 95°C denature for 5min, followed by 40 cycles each containing 95°C for 15sec and 60°C for 1min. PCR was performed on Bio-Rad CFX96 real-time fluorescent quantitative PCR cycler for data storage.

Western Blot for protein expression assay

Total proteins were extracted from cells or tissues by RIPA lysis buffer. Protein concentration and quality were measured by BCA approach. 40μg samples were loaded for separation in SDS-PAGE (10% separating gel and 4% condensing gel), and were transferred to PVDF membrane (300mA, 90min). The membrane was blocked in PBST containing 5% defatted milk powder for 60min at room temperature. Primary antibody (anti-Nrf2 at 1:1000, anti-HO-1 at 1:2000, anti-β-actin anti 1:10000) was added for 4°C overnight incubation. PBST rinsing was performed for three times to remove unbounded primary antibody. HRP conjugated goat anti-rabbit IgG (H+L) secondary antibody at 1:10000 dilution was added for 60min room temperature incubation. After PBST rinsing for three times, BeyoECL Plus working solution A and solution B were mixed in equal volumes. BeyoECL Plus working solution was added onto the protein blotting membrane. After 2-3 min dark incubation, the membrane was exposed, developed, and the film was scanned for storage for data.

STATISTICAL ANALYSIS

SPSS 18.0 was used for data analysis. Measurement data were presented as mean±standard deviation (SD). Comparison of measurement data between two groups was performed by student t-test. Comparison of measurement data among multiple groups was performed by one-way analysis of variance (ANOVA), followed by Bonferroni comparison between two groups. A statistical significance was defined when $P<0.05$.

RESULTS

Prominent neurological dysfunction of SAE model mice

Mice received CLP surgery presented sepsis symptoms including piloerection, shivering, immobility, lethargy, or even bleeding of nose-eye in severe conditions. Within 24h observation window, Sham group had no mortality, and 8 out of 60 CLP model mice died. In the remaining 32 survived mice, 14 of them did not present SAE and 18 of

them showed symptoms. Sham mice had satisfactory mental status and basically normal neural reflex. SAE mice showed remarkably weakened neural reflex function and lower score (table 1).

Enhanced OS level and cell apoptosis of SAE model mouse brain tissues

Test results showed that, comparing to Sham group, SAE model mice had significantly elevated caspase-3 activity in brain tissues (fig. 1A). Flow cytometry results showed remarkably higher apoptotic rate in SAE mouse brain tissues comparing to Sham group (fig. 1B). Using quantification kit, we found significantly higher MDA content in SAE model mouse brain tissues comparing to Sham group (fig. 1C), whilst enzymatic activity of SOD (fig. 1D) and GSH-Px (fig. 1E) were significantly decreased. ELISA results showed that comparing to Sham mouse, SAE model had remarkably elevated S100β content in peripheral blood samples (fig. 1F).

Rg1 in alleviating brain OS level and cell apoptosis in SAE mouse brain tissues

Test results showed that comparing to SAE mouse, Rg1 treatment significantly depressed caspase-3 activity in brain tissues, with more potent depression in high-dosage group (fig. 2A). Flow cytometry showed significantly decreased apoptotic rate in Rg1 treated mouse brain tissues comparing to SAE group, with even lower apoptotic rate in high-dosage group (fig. 2C). Using test kit, MDA content in Rg1 treated mouse brain tissues was remarkably decreased comparing to SAE group (fig. 2B), whilst SOD enzymatic activity was significantly increased (fig. 2D). ELISA also found prominently suppressed S100β concentration in peripheral blood of Rg1 treated mice comparing to SAE group (fig. 2E).

Test for neural reflex function showed significantly improved neural reflex score in Rg1 treatment group comparing to SAE group, with even higher score in high-dosage group than low-dosage group (table 2).

Rg1 up-regulation of Nrf2 and HO-1 expression in SAE mouse brain tissues

qRT-PCR results showed that comparing to SAE group, Rg1 treatment significantly elevated brain expression of Nrf2, HO-1 mRNA, with higher expression in Rg1 high-dosage group than low-dosage treatment group (fig. 3A). Western blot results also showed remarkably elevated Nrf2 and HO-1 protein expression in Rg1 treated mice comparing to SAE group, with more potent effects in high-dosage group comparing to low dosage group (fig. 3B).

Rg1 alleviates MN-c cell apoptosis and ROS production via enhancing Nrf2 and HO-1

ELISA results showed significantly elevated contents of TNF-α, IL-1β and IL-6 in supernatant of LPS-treated BV2 cells, and Rg1 treatment remarkably inhibited the release of TNF-α (fig. 4), IL-1β (fig. 4B) and IL-6 (fig. 4C) in culture medium. Flow cytometry results indicated significantly elevated Iba-1 positive expression rate in LPS-treated BV2 cells comparing to control group, indicating LPS-induced microglia activation. Rg1 treatment down-regulated Iba-1 and inhibited microglia activation (fig. 4D).

qRT-PCR results showed that comparing to control group, LPS treated MN-c cells had remarkably elevated Nrf2 and HO-1 mRNA expression. Rg1 treatment further enhanced Nrf2 and HO-1 mRNA expression (fig. 5A). Western blot results showed that comparing to control group, LPS treated MN-c cells had significantly enhanced expression of Nrf2 and HO-1 proteins. Rg1 treatment further enhanced protein expression of Nrf2 and HO-1 (fig. 5B). Flow cytometry revealed that comparing to control group, LPS treated MN-c cells had significantly elevated ROS content (fig. 5C), plus remarkably enhanced cell apoptosis (fig. 5D). Rg1 treatment remarkably suppressed ROS production or cell apoptosis of LPS-treated MN-c cells.

DISCUSSION

Oxidative stress (OS) refers to the breakdown of endogenous oxidation/anti-oxidation homeostasis as the consequence of environmental stimuli and belongs to one type of body stress response. Under physiological conditions, a dynamic balance exists between body anti-oxidation and oxidation systems, which efficiently clears ROS free radicals including H_2O_2, hydroxyl radicals and superoxide anions. Under the existence of over-stimuli by environment or abnormal body status, the over-production of ROS and/or impaired clearance capacity of ROS result in OS condition. ROS can directly or indirectly damage physiological functions of macromolecules including protein, lipid and nucleic acid, and is thus physiological and pathological basis for various diseases.

The production of large amounts of ROS can cause endoplasmic reticular stress, mitochondrial dysfunction, calcium homeostatic disruption of brain cells, leading to apoptosis. ROS induced apoptosis plays crucial roles in onset and progression of SAE (Ji *et al.*, 2018; Kawakami *et al.*, 2018; Kuperberg and Wadgaonkar, 2017; Molnar *et al.*, 2018; Wu *et al.*, 2015). Body has evolved one complicated system of OS response and can induce a series of protective proteins under the exposure of ROS to relieve cell damage. Nrf2/antioxidant response element (AR) is the most important endogenous anti-OS signal pathway ever been found. Under OS condition, Nrf2 can translocate into the nucleus to bind with ARE for initiating transcription and expression of downstream phase II detoxifying enzyme, anti-oxidase, and anti-apoptotic genes, thus potentiating cellular resistance against OS stress of large electrophilic biomolecules, alleviating body OS injury or cell apoptosis, thus

protecting pathological tissues or cells (Luo *et al.*, 2011; Tabei *et al.*, 2017; Wang *et al.*, 2017). Under OS condition, Keap1 has structural changes to release Nrf2 for nuclear translocation and binding with ARE, resulting in the up-regulation of various anti-oxidative proteins including HO-1, γ-GCS, SOD, GST and NAD(P)H, thus relieving OS level and decreasing OS injury (David *et al.*, 2017; Keleku-Lukwete *et al.*, 2017; Zhang *et al.*, 2017; Zhu *et al.*, 2017).

Ginsenoside is one group of steroid compound and mainly exists in ginseng, with multiple pharmaceutical activities including anti-oxidation, anti-aging, anti-inflammation, decreasing cell apoptosis and neuronal protection (Jin *et al.*, 2017; Li *et al.*, 2016; Wang *et al.*, 2018). Ginsenoside plays important protective roles in sepsis, including anti-inflammation, anti-oxidation and maintenance of mitochondrial functional stability (Xing *et al.*, 2017; Zou *et al.*, 2013). However, few studies have been performed regarding the role of Ginsenoside in SAE. This study thus generated an SAE mouse model, on which different dosage of Ginsenoside was applied to investigate its role in alleviating SAE brain damage, suppressing OS and brain cell apoptosis.

Results from this study showed that comparing to Sham group, SAE rats showed significantly weakened neural reflex functions, as indicated by lower score, suggesting prominent brain dysfunction and successful generation of SAE model. Test results showed significantly elevated MDA content in SAE mouse brain tissues, enhanced caspase-3 activity and cell apoptotic rate, and depressed activity of SOD or GSH-Px, indicating remarkably elevated OS level in brain levels plus decreased anti-oxidation potency, leading to higher cell apoptosis. S100β is one type of calcium binding protein and mainly exists in brain glial cells with relatively higher brain specificity. Under the brain tissue injury or BBB destruction, S100β can penetrate BBB to enter the blood circulation, thus alleviating its serum levels. Therefore, serum assay for S100β is one common index evaluating brain damage (Engoren *et al.*, 2017; Kuriyama *et al.*, 2016; Yoshii *et al.*, 2018). This study showed that comparing to Sham group, SAE mice showed significantly elevated serum S100β level, indicating brain tissue damage. Rg1 treatment on SAE mice decreased brain MDA level, increased anti-oxidase SOD activity, suppressed caspase-3 activity or apoptosis rate, and suppressed serum S100β levels. Rg1 high-dosage group had more potent effects than Rg1 low-dosage group in the sense of these phenotypes. These results indicate that Rg1 can potentiate anti-oxidation ability of SAE mouse brain tissue, decrease OS level or apoptotic rate, and alleviates brain tissue damage. Due to the critical role of Nrf2 in responding OS and initiating anti-OS mechanism, we measured the expression of Nrf2 and downstream anti-oxidative factors. Results showed that comparing to SAE group, Rg1 treated mice showed remarkably elevated Nrf2 and HO-1 mRNA/protein levels. With higher Rg1 dosage, Nrf2 and HO-1 expression levels were further enhanced. These results supported that Rg1 could activate Nrf2/ARE signal pathway via up-regulating Nrf2 expression, thus enhancing mouse brain anti-oxidation mechanism and level, alleviating brain OS level, cell apoptosis or brain damage. LPS treatment activated *in vitro* culture of microglia cells, which were co-cultured with neurons to mimic *in vivo* condition of SAE. Results showed that LPS treatment up-regulated Iba-1 expression in BV2 cells, stimulated the expression of inflammatory factors TNF-α, IL-1β and IL-6, and remarkably induced MN-c neuron apoptosis or ROS production. Rg1 treatment significantly inhibited the release of TNF-α, IL-1β and IL-6, or Iba-1 expression, and decreased MN-c cell apoptosis and ROS production. The alleviation of MN-c cell apoptosis or ROS production by Rg1 is correlated with Nrf2 or HO-1 up-regulation.

In a correlation study between Ginsenoside and sepsis, a previous study found that Ginsenoside can enhance anti-OS potency, alleviate mitochondrial dysfunction, ROS production or cell apoptosis via modulating AMPK pathway, to suppress sepsis induced liver tissue damage or *in vitro* injury of stem cells (Xing *et al.*, 2017). In addition, Ginsenoside can relieve pulmonary damage of sepsis model rats, and inhibited the production of inflammatory factors TNF-α, MCP-1 and IL-8, or infiltration of inflammation cells (Yuan *et al.*, 2014). Furthermore, Ginsenoside is reported to remarkably inhibit inflammatory response in septic mice, and relieve liver or pulmonary damage (Zou *et al.*, 2013). Meanwhile, Ginsenoside could also reduce the release of inflammatory factors and cell apoptosis from SAE mouse brain tissues via suppressing hippocampal cell autophagy (Li *et al.*, 2017). In this study, Ginsenoside can relieve SAE pathology, as similar with Li et al (Li *et al.*, 2017).

We further revealed the role of Ginsenoside in alleviating SAE brain damage, via functional mechanisms related with Nrf2 up-regulation, enhancement of neuron anti-oxidation potency, and reduction of cell apoptosis, which have not been reported in a previous study (Li *et al.*, 2017). However, this study also observed that Ginsenoside could inhibit microglial activation or release of inflammatory factors, probably related with its anti-inflammatory functions, leaving its detailed mechanisms unclear that requires further studies to satisfy the weakness of the current work.

CONCLUSION

In SAE mouse model, Ginsenoside can enhance anti-OS potency of brain tissues, decrease brain OS damage or cell apoptosis via up-regulating Nrf2 and HO-1 expression, thus alleviating SAE brain damage.

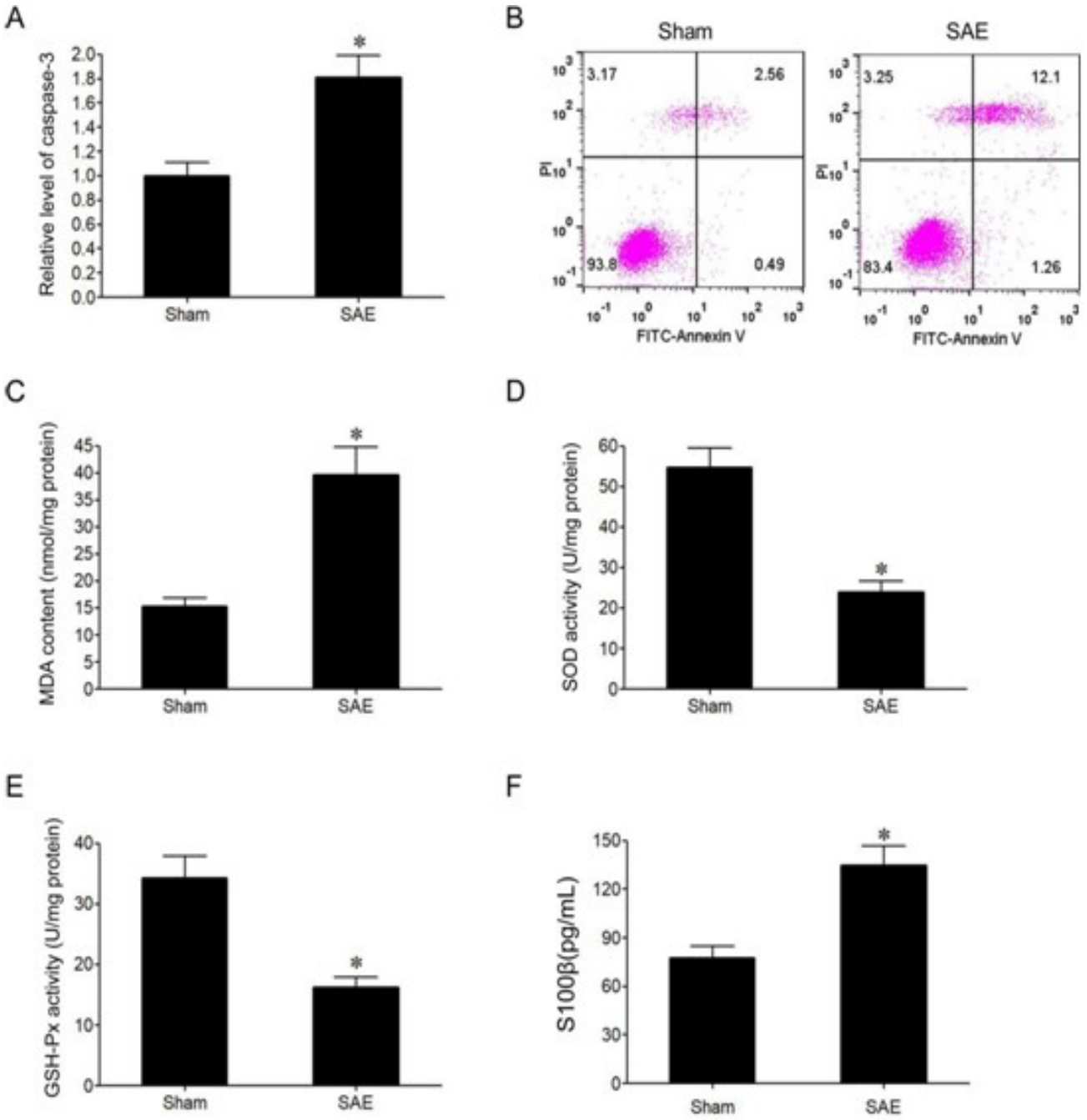

Fig. 1: Elevated OS level and cell apoptosis level in SAE mouse brain tissues. (A) Caspase-3 activity in mouse brain tissues by test kit. (B) Flow cytometry assay for brain cell apoptosis. (C) MDA content in mouse brain tissues by test kit. (D) SOD enzymatic activity in mouse brain tissues. (E) GSH-Px enzymatic activity in mouse brain by test kit. (F) ELISA for serum S100β concentration. *, $P<0.05$ comparing to Sham group using student t-test. SAE: sepsis-associated encephalopathy; SOD: super oxide dismutase.

Table 1: Comparison of neural reflex function score between two groups of mice after surgery

Group	Neural reflex function score
Sham group	9.53±0.41
SAE group	5.27±0.49*

*, $P<0.05$ comparing to Sham group using student t-test. SAE: sepsis-associated encephalopathy.

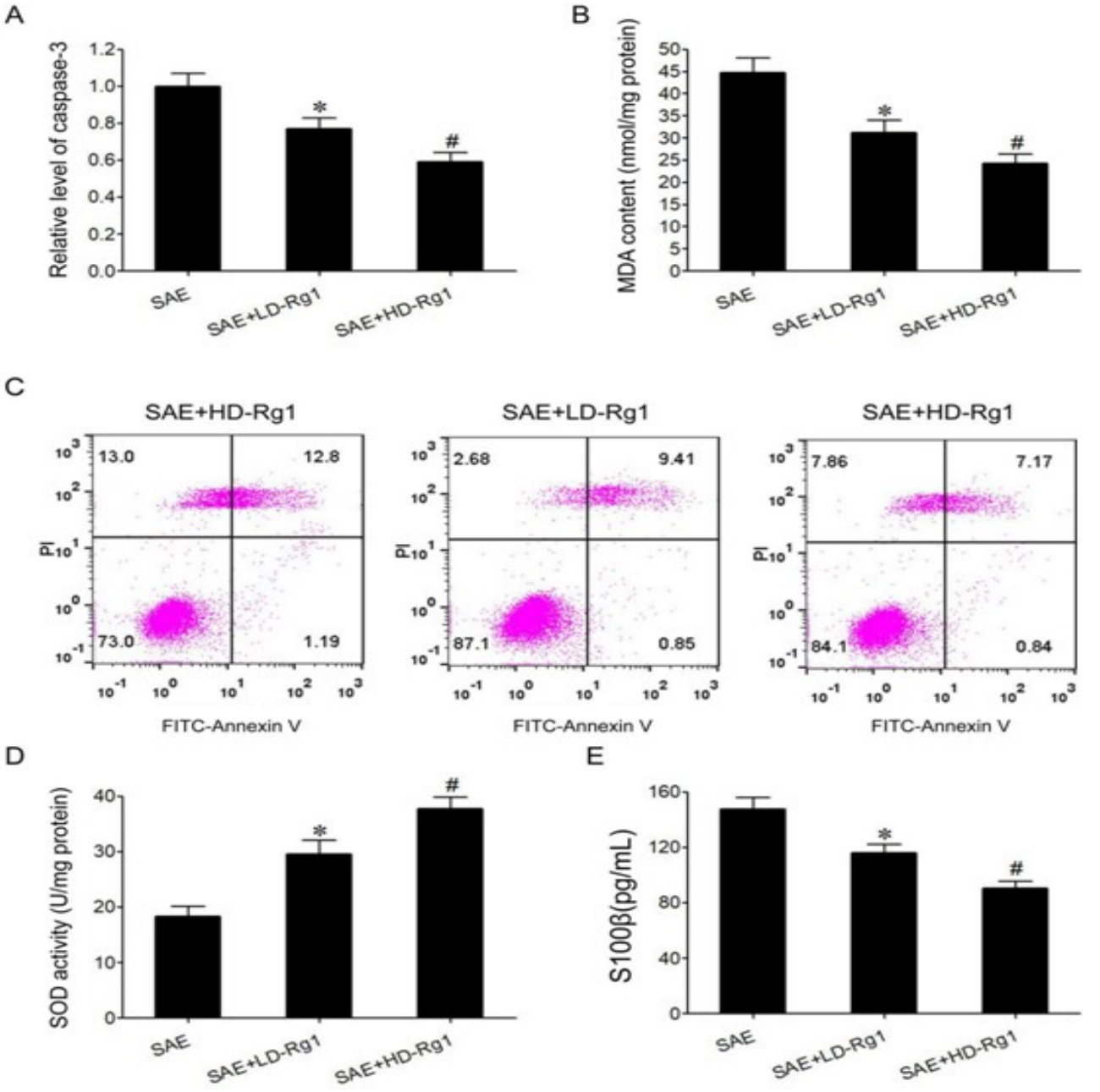

Fig. 2: Elevated OS level and apoptosis in SAE mouse brain tissues. (A) Test kit for caspase-3 activity in mouse brain tissues. (B) MDA content in mouse brain tissues by test kit. (C) Flow cytometry assay for brain cell apoptosis. (D) SOD enzymatic activity in mouse brain tissues. (E) ELISA for serum S100β concentration. *, $P<0.05$ comparing to SAE group; #, $P<0.05$ comparing to SAE+LD-Rg1 group using one-way ANOVA. Rg1: Ginsenoside; SAE: sepsis-associated encephalopathy; OS: oxidative stress; MDA: malondialdehyde; SOD: superoxide dismutase.

Table 2: Comparison of neural reflex function scores among all groups of mice

Group	Neural reflex function score
SAE	5.16±0.43
SAE+LD-Rg1	6.27±0.46*
SAE+HD-Rg1	7.33±0.52#

*, $P<0.05$ comparing to SAE group.; #, $P<0.05$ comparing to SAE+LD-Rg1 group using one-way ANOVA. Rg1: Ginsenoside; SAE: sepsis-associated encephalopathy.

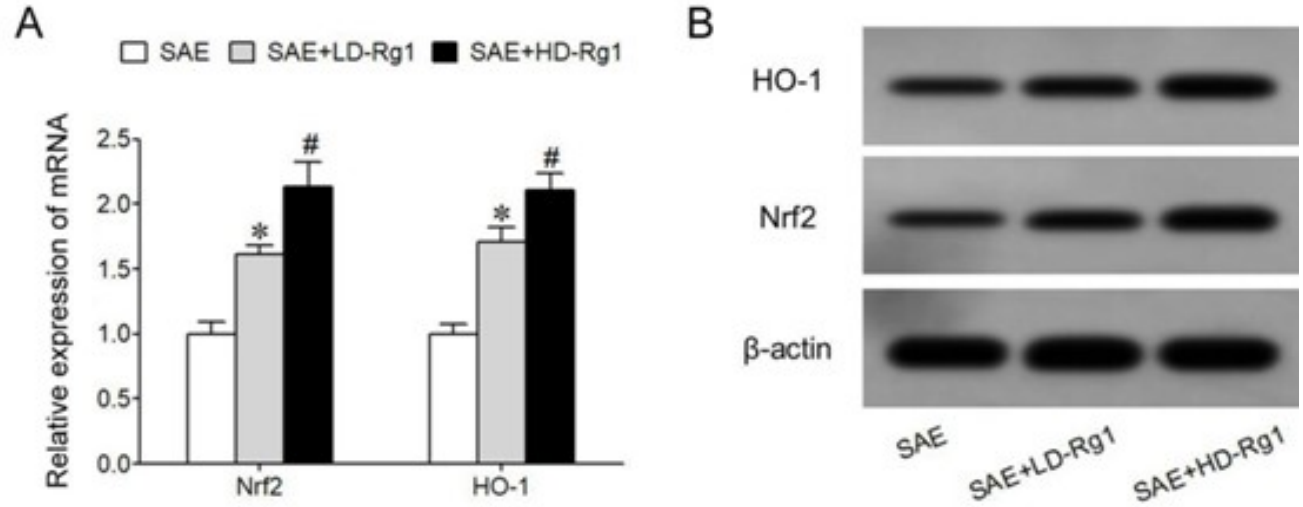

Fig. 3: Rg1 up-regulates Nrf2 and HO-1 expression in SAE mouse brain tissues. (A)qRT-PCR for mRNA expression in mouse brain tissues. (B) Western blot for measuring protein expression in mouse brain tissues. *, P<0.05 comparing to SAE group.; #, P<0.05 comparing to SAE+LD-Rg1 group using one-way ANOVA. SAE: sepsis-associated encephalopathy.

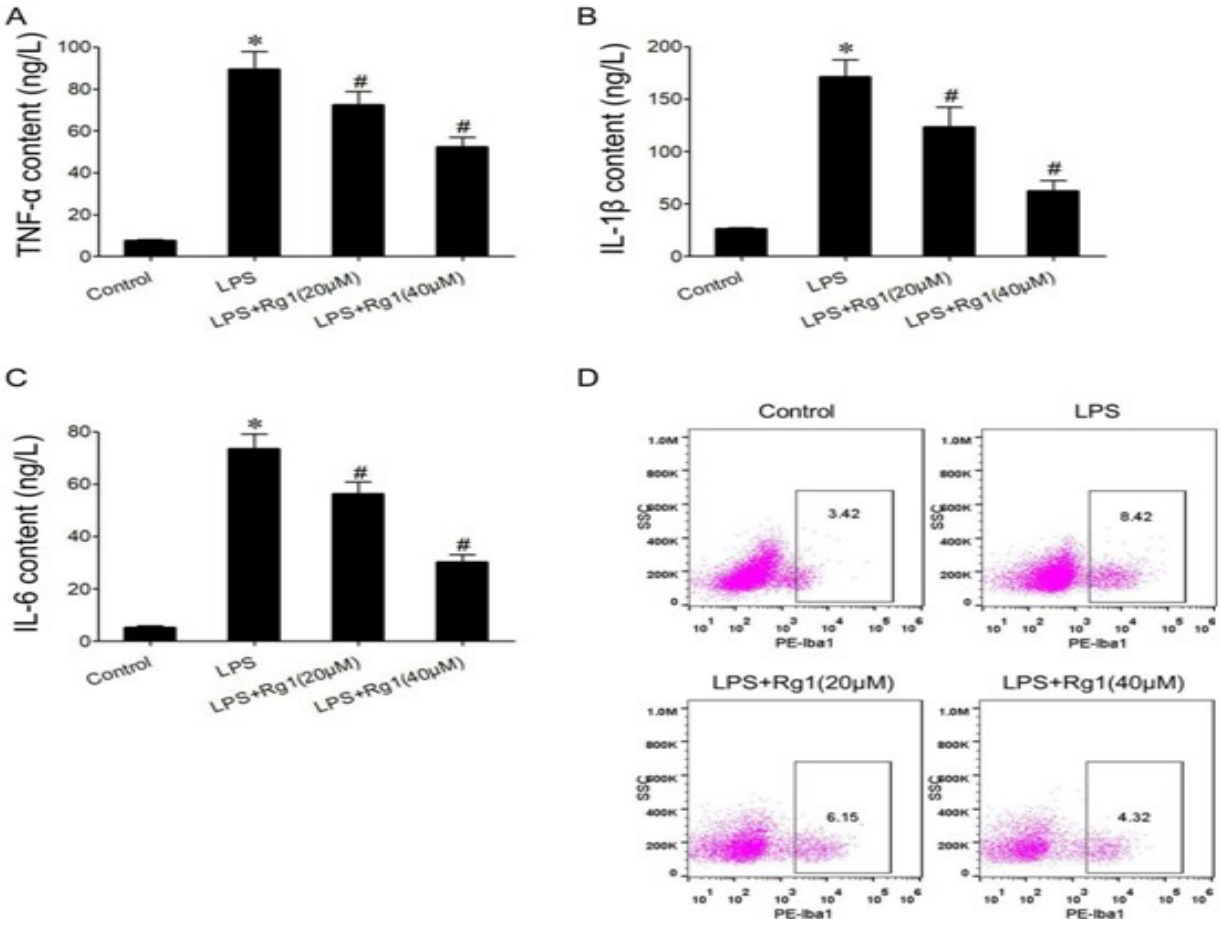

Fig. 4: Rg1 inhibited LPS induced microglial activation. (A) ELISA for measuring TNF-α content. (B) ELISA for testing IL-1β level. (C) ELISA for measuring IL-6 concentration. (D) Flow cytometry measuring Iba-1 expression. *, P<0.05 comparing to control group.; #, P<0.05 comparing to LPS group using one-way ANOVA. Rg1: Ginsenoside; SAE: sepsis-associated encephalopathy.

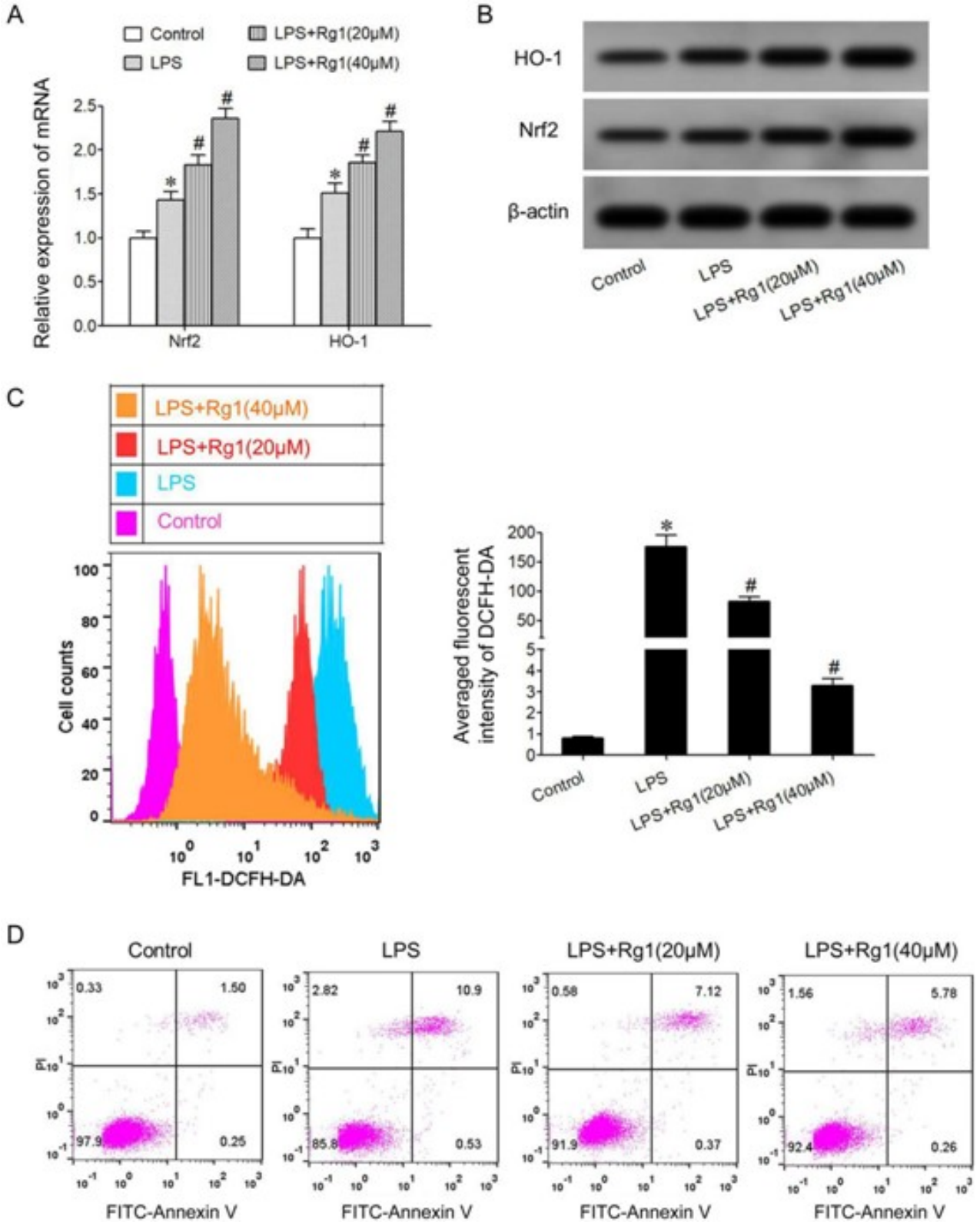

Fig. 5: Rg1 alleviates MN-c cell apoptosis or ROS production via up-regulating Nrf2 and HO-1. (A) qRT-PCR for Nrf2 and HO-1 mRNA expression in MN-c cells. (B) Western blot assay for Nrf2 and HO-1 protein expression in MN-c cells. (C) Flow cytometry for ROS content in MN-c cells. (D) Flow cytometry measuring MN-c cell apoptosis. *, $P<0.05$ comparing to control group; #, $P<0.05$ comparing to LPS group using one-way ANOVA. Rg1: Ginsenoside.

REFERENCES

Caserta S, Kern F, Cohen J, Drage S, Newbury SF and Llewelyn MJ (2016). Circulating plasma microRNAs can differentiate human sepsis and systemic inflammatory response syndrome (SIRS). *Sci. Rep.,* **6**: 28006.

Chaudhry N and Duggal AK (2014). Sepsis Associated Encephalopathy. *Adv Med.,* 762320.

Cotena S and Piazza O (2012). Sepsis-associated encephalopathy. *Transl. Med. UniSa.,* **18**(2): 20-27.

David JA, Rifkin WJ, Rabbani PS and Ceradini DJ (2017). The Nrf2/Keap1/ARE pathway and oxidative stress as a therapeutic target in type II diabetes mellitus. *J Diabetes Res.,* 4826724.

Engoren M, Brown RR and Dubovoy A (2017). A retrospective analysis of the effect of blood transfusion on cerebral oximetry entropy and acute kidney injury. *Perfusion,* **32**(1): 35-43.

Feng Q, Ai YH, Gong H, Wu L, Ai ML, Deng SY, Huang L, Peng QY and Zhang LN (2017). Characterization of sepsis and sepsis-associated encephalopathy. *J Intensive Care Med.,* **34**(11-12): 938-945

Ji MH, Xia DG, Zhu LY, Zhu X, Zhou XY, Xia JY and Yang JJ (2018). Short- and long-term protective effects of melatonin in a mouse model of sepsis-associated encephalopathy. *Inflammation,* **41**(2): 515-529.

Jiang W, Wang Z, Jiang Y, Lu M and Li X (2015). Ginsenoside Rg1 ameliorates motor function in an animal model of parkinson's disease. *Pharmacology,* **96**(1-2): 25-31.

Jin Y, Peng J, Wang X, Zhang D and Wang T (2017). Ameliorative effect of ginsenoside Rg1 on lipopolysaccharide-induced cognitive impairment: role of cholinergic system. *Neurochemical Research,* **42**: 1299-1307.

Kawakami M, Hattori M, Ohashi W, Fujimori T, Hattori K, Takebe M, Tomita K, Yokoo H, Matsuda N, Yamazaki M and Hattori Y (2018). Role of G protein-coupled receptor kinase 2 in oxidative and nitrosative stress-related neurohistopathological changes in a mouse model of sepsis-associated encephalopathy. *Journal of Neurochemistry*, **145**(6): 474-488.

Keleku-Lukwete N, Suzuki M and Yamamoto M (2017). An overview of the advantages of KEAP1-NRF2 system activation during inflammatory disease treatment. *Antioxidants & Redox Signaling*, **29**(17): 1746-1755.

Kuperberg SJ and Wadgaonkar R (2017). Sepsis-associated encephalopathy: the blood-brain barrier and the sphingolipid *Rheostat. Front Immunol.,* **156**(8): 597.

Kuriyama N, Mizuno T, Yasuike H, Matsuno H, Kawashita E, Tamura A, Ozaki E, Matsui D, Watanabe I, Koyama T, Miyatani F, Kondo M, Tokuda T, Ohshima Y, Muranishi M, Akazawa K, Takada A, Takeda K, Matsumoto S, Mizuno S, Yamada K, Nakagawa M and Watanabe Y (2016). CD62-mediated activation of platelets in cerebral white matter lesions in patients with cognitive decline. *Arch. Gerontol. Geriatr.,* **62**: 118-124.

Li F, Wu X, Li J and Niu Q (2016). Ginsenoside Rg1 ameliorates hippocampal long-term potentiation and memory in an Alzheimer's disease model. *Mol Med Rep.,* **13**(6): 4904-4910.

Li Y, Wang F and Luo Y (2017). Ginsenoside Rg1 protects against sepsis-associated encephalopathy through beclin 1-independent autophagy in mice. *The Journal of Surgical Research,* **207**: 181-189.

Luo C, Urgard E, Vooder T and Metspalu A (2011). The role of COX-2 and Nrf2/ARE in anti-inflammation and antioxidative stress: Aging and anti-aging. *Med. Hypotheses.,* **77**(2): 174-178.

Molnar L, Fulesdi B, Nemeth N and Molnar C (2018). Sepsis-associated encephalopathy: A review of literature. *Neurol. India,* **66**(2): 352-361.

Tabei Y, Murotomi K, Umeno A, Horie M, Tsujino Y, Masutani B, Yoshida Y and Nakajima Y (2017). Antioxidant properties of 5-hydroxy-4-phenyl-butenolide via activation of Nrf2/ARE signaling pathway. *Food Chem. Toxicol.,* **107**(Part A): 129-137.

Wang K, Chen Z, Huang L, Meng B, Zhou X, Wen X and Ren D (2017). Naringenin reduces oxidative stress and improves mitochondrial dysfunction via activation of the Nrf2/ARE signaling pathway in neurons. *Int. J. Mol. Med.,* **40**(5): 1582-1590.

Wang L, Zhao H, Zhai ZZ and Qu LX (2018). Protective effect and mechanism of ginsenoside Rg1 in cerebral ischaemia-reperfusion injury in mice. *Biomed. Pharmacother.,* **99**: 876-882.

Wu J, Zhang M, Hao S, Jia M, Ji M, Qiu L, Sun X, Yang J and Li K (2015). Mitochondria-targeted peptide reverses mitochondrial dysfunction and cognitive deficits in sepsis-associated encephalopathy. *Mol. Neurobiol.,* **52**: 783-791.

Xing W, Yang L, Peng Y, Wang Q, Gao M, Yang M and Xiao X (2017). Ginsenoside Rg3 attenuates sepsis-induced injury and mitochondrial dysfunction in liver via AMPK-mediated autophagy flux. *Bioscience Reports,* **37**(4): BSR20170934.

Xu C, Guo Z, Zhao C, Zhang X and Wang Z (2018). Potential mechanism and drug candidates for sepsis-induced acute lung injury. *Exp. Ther. Med.,* **15**(6): 4689-4696.

Yamaga S, Shime N, Sonneville R, de Montmollin E and Timsit JF (2017). Risk factors for sepsis-associated encephalopathy. *Intensive Care Med.,* **43**(10): 1548-1549.

Yang Y, Li X, Zhang L, Liu L, Jing G and Cai H (2015). Ginsenoside Rg1 suppressed inflammation and neuron apoptosis by activating PPARgamma/HO-1 in hippocampus in rat model of cerebral ischemia-reperfusion injury. *Int J Clin Exp Pathol.,* **8**(3): 2484-2494.

Yoshii F, Kawaguchi C, Kohara S, Shimizu M, Onaka H, Ryo M and Takahashi W (2018). Characteristic deterioration of ADAS-Jcog subscale scores and correlations with regional cerebral blood flow reductions in Alzheimer's disease. *Neurol. Sci.,* **39**: 909-918.

Yuan Q, Jiang YW, Ma TT, Fang QH and Pan L (2014). Attenuating effect of ginsenoside Rb1 on LPS-induced lung injury in rats. *J. Inflamm.* (Lond)., **11**(1): 40.

Zhang JC, Yao W, Dong C, Han M, Shirayama Y and Hashimoto K (2017). Keap1-Nrf2 signaling pathway confers resilience versus susceptibility to inescapable electric stress. *Eur. Arch. Psychiatry Clin. Neurosci.,* **268**: 865-870.

Zhang LN, Wang XT, Ai YH, Guo QL, Huang L, Liu ZY and Yao B (2012). Epidemiological features and risk factors of sepsis-associated encephalopathy in intensive care unit patients: 2008-2011. *Chin. Med. J.,* **125**(5): 828-831.

Zhu C, Dong Y, Liu H, Ren H and Cui Z (2017). Hesperetin protects against H2O2-triggered oxidative damage via upregulation of the Keap1-Nrf2/HO-1 signal pathway in ARPE-19 cells. *Biomed. Pharmacother.,* **88**: 124-133.

Zou Y, Tao T, Tian Y, Zhu J, Cao L, Deng X and Li J (2013). Ginsenoside Rg1 improves survival in a murine model of polymicrobial sepsis by suppressing the inflammatory response and apoptosis of lymphocytes. *J. Surg. Res.,* **183**(2): 760-766.

Zrzavy T, Hoftberger R, Berger T, Rauschka H, Butovsky O, Weiner H and Lassmann H (2018). Pro-inflammatory activation of microglia in the brain of patients with sepsis. *Neuropathol. Appl. Neurobiol.,* **45**(3): 278-290