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Glycyrrhizin suppresses the expressions of HMGB1 and ameliorates inflammative effect after acute subarachnoid hemorrhage in rat model

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ABSTRACT

High-mobility group box 1 (HMGB1), a nuclear protein that has endogenous cytokine-like activity, is involved in early brain injury after subarachnoid hemorrhage (SAH) by mediating inflammatory response. This study was conducted to investigate the effect of glycyrrhizin as an inhibitor of HMGB1 in a rat SAH model. Experimental SAH was induced by using autologous blood injection to prechiasmatic cistern. 15 mg/kg glycyrrhizin was administered immediately after SAH induction, and then administered once at 6, 12 and 18 h. All the rats were sacrificed at 24 h after neurological assessment and frontal brain tissue was taken for assay. Blood–brain barrier (BBB) permeability was determined by Evans blue (EB) extravasation. The expression of HMGB1 were detected by immunofluorescence, western blot and quantitative real-time PCR. Inflammatory mediators (TNF-α, IL-1β) were measured using specific ELISA. Fluoro-Jade C staining and TUNEL staining was performed for the quantitative assessment of neuronal injury. We found the use of glycyrrhizin significantly improved neurological scores, reduced HMGB1-positive cells, down-regulated mRNA and protein levels of HMGB1, inhibited BBB permeability, and attenuated neuronal cell death and apoptosis after SAH. The up-regulations of inflammation-related molecules (TNF-α, IL-1β) in SAH rats were suppressed by glycyrrhizin treatment. These findings suggest that glycyrrhizin is a potential candidate for the treatment of inflammatory brain injury after SAH.

1. Background

Subarachnoid hemorrhage (SAH) is a life-threatening disease. The outcome of patients after SAH is generally poor: about one-half of patients died and many survivors have long-term cognitive and neurological impairment [1]. Vasospasm and early brain injury (EBI) are considered as main courses of the poor outcome after SAH, and overwhelming evidence indicates that inflammation plays a crucial role in both processes [2,3].

High-mobility group box 1 (HMGB1) is a highly conserved, ubiquitous protein expressed in the nuclei of nearly all cell types. It plays a pivotal role in bending DNA, stabilizing nucleosome formation and regulating gene transcription. In various pathological states, HMGB1 could be passively released from necrotic cells or actively secreted from immune cells or non-immune parenchymal cells [4,5]. Extracellular HMGB1 serves as a damage-associate molecular pattern (DAMP) through binding to the receptor for advanced glycation end-products (RAGE) or toll-like receptor (TLR)-2 and TLR-4 receptors, which activate pro-inflammatory pathways and aggravate the inflammatory injury [4,6,7]. Based on recent studies, the level of HMGB1 was significantly up-regulated and associated with poor functional outcome and mortality after SAH [8]. It is believed that triggered inflammation and contributed to the EBI after SAH [9].

Glycyrrhizin (GL) is a triterpenoid saponin compound produced by the root of the licorice plant, Glycyrrhiza glabra. Recently, GL was recognized as an HMGB1 inhibitor, which can bind directly to HMGB1 and inhibit its extracellular cytokine activities [10–12]. Several studies indicated that the treatment with GL can alleviate inflammation and brain injury in cerebral ischemia, intracerebral hemorrhage (ICH) and traumatic brain injury (TBI) animal models [11,13–15]. However, the roles and mechanisms of GL in the treatment of SAH, especially HMGB1-induced inflammation and brain injury after SAH, were not investigated previously [10]. Therefore, this study was conducted to investigate the effect of GL as an inhibitor of HMGB1 in rat SAH model.

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2. Method

2.1. Animals and animal groups

Male Sprague–Dawley rats weighing 260–310 g were purchased from the Experimental Animal Center of Sichuan University, Chengdu, China. Animals were treated in accordance with the guidelines for animal research of Sichuan University, Chengdu, China and the animal experimental protocol was approved by Ethics Committee. Rats were randomly divided into the following three groups (n = 25 in each group): (1) sham operated group (no SAH); (2) SAH group, in which SAH was induced and rats received 0.9% saline instead of GL at each of the time points. (3) SAH + GL group, in which SAH was induced, and then GL 15 mg/kg (Biopurify Phytochemicals Ltd., Chengdu, China) was administered by intraperitoneal injection immediately, and then administered once at 6, 12 and 18 h after SAH induction. All the rats were killed at 24 h to collect samples after neurological assessment. Six rats in each group were sacrificed for western blot, enzyme-linked immunosorbent assay and Quantitative Real-time PCR. Six rats in each group were sacrificed for immunofluorescence, TUNEL assay and FJC staining. Six rats in each group were for detecting blood–brain barrier (BBB) impairment.

2.2. SAH model

Experimental SAH model was induced by using stereotaxic autologous blood injection to prechiasmatic cistern as reported previously [16,17]. Rats were anesthetized by intraperitoneal injection of 4% chloral hydrate (1 ml/100 g). After stereotactic fix, skin incision and preparation of the skull, a needle was tilted 45° in the sagittal plane and placed 7.5 mm anterior to bregma in the midline, with the hole facing the right side. It was lower until the tip reached the base of the skull, about 2–3 mm anterior to the chiasma (about 10–12 mm away from the brain surface), and then retracted 0.5 mm. Before inserting the needle, bone wax was used to plugging the burr hole for the prevention of cerebrospinal fluid loss and bleeding from the midline vessels. Then 0.25 ml non-heparinized fresh autologous arterial blood was taken from the femoral artery and slowly injected into the prechiasmatic cistern for 20 s with a syringe pump. Control rats were injected with 0.25 ml saline.

2.3. Neurological scoring

Sensorimotor deficits of SAH rats in each group were evaluated at 24 h with a modification of 18-point scoring system reported by Garcia et al. [18]. The scoring system was elaborated in Appendix A.

2.4. Blood–brain barrier permeability

Extravasation of Evans blue (EB) were performed at 24 h to determine blood–brain barrier permeability as described methods in previous report [19,20]. Briefly, rats were intravenously injected with EB at a dose of 100 μg/mg. Rats were then re-anesthetized after 1 h and perfused using saline. After brain removed and homogenized in phosphate-buffered saline, Trichloroacetic acid was added to precipitate protein, and the samples were cooled and centrifuged. The resulting supernatant was measured for absorbance of EB using a spectrophotometer at 620 nm.

2.5. Immunofluorescence, TUNEL assay and FJC staining

Rats were perfused with saline followed by 4% buffered paraformaldehyde at 24 h after SAH induction. The brains were immersed in 4% buffered paraformaldehyde overnight and then the most representative fields (frontal brain tissue adjacent to SAH) (Fig. 1) were selected for additional cuts of frozen sections. Frozen sections (4μm thick) were incubated overnight at 4 °C with primary antibodies (Abcam) against HMGB1. Then sections were incubated with anti-rabbit secondary antibody for 2 h at room temperature. Nuclei were counterstained using 4′,6-diamidino-2-phenylindole (DAPI). Sections were observed by a fluorescence microscope (Olympus) and analyzed using Image J 1.48 (U.S. National Institutes of Health).

Fluoro-Jade C (FJC) staining and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed for the quantitative assessment of neuronal injury after SAH. Pre-staining procedures of a series of frozen sections were the same. Staining was performed according to the manufacturers' instructions.

The number of HMGB1, TUNEL and FJC positive cells was presented as the percentage of total cells in each visual field counted in 5 randomly microscopic fields of each sample (at 200× magnifications).

2.6. Western blot analysis

Rats in each group were euthanized by intracardiac perfusion with saline through the left cardiac ventricle until effluent from the right atrium was clear. The frozen brain samples of frontal brain tissue was removed and stored at −80 °C till further use for western blot and real-time PCR. Tissue samples were homogenized and extracted, proteins were separated by 10–12% SDS–polyacrylamide gel electrophoresis. Fractionated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were incubated with primary anti-bodies against HMGB1 (Abcam), followed by incubation with homologous secondary antibodies (Santa Cruz). Protein bands were visualized by enhanced chemiluminescence, and signal intensity was quantitated using Image J 1.48 software. HMGB1 protein levels were normalized to β-actin levels.

2.7. Quantitative Real-time PCR

Quantitative Real-time PCR was used to analyze the mRNA levels of HMGB1. Total RNA was extracted with Trizol reagent (Invitrogen Corporation, Carlsbad, CA, USA) following the manufacturer's instructions. Isolated RNA was reverse transcribed into cDNA using Promega (Madison, WI, USA) reagents. Real-time PCR analysis proceeded by real-time DNA analysis system (Genetimes Technology, Inc., Shanghai, China), using real-time SYBR Green PCR technology. The primers were synthesized by Life Technologies (Invitrogen, Shanghai, China) and the primers sequences used to determine HMGB1 gene expressions were: forward (F): 5′-TCC TTCGGCCTTCTTCTTGT-3′, reverse (R): 5′-CGGCCTTCTTTTCA TAGGGC-3′.

2.8. Enzyme-linked immunosorbent assay (ELISA)

The dissociation and protein extraction procedure for frontal brain tissue were the same to that in western blot. Inflammatory mediators tumor necrosis factor (TNF)-α and Interleukin (IL)-1β were measured using specific ELISA kits for rats according to the manufacturer's instructions (Biosource, Camarillo, CA).

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