

## Research Article

# Glycyrrhizin Attenuates Toll Like Receptor-2, -4 and Experimental Vasospasm in a Rat Model

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Upregulated TLRs are observed in the serum of animals following experimental subarachnoid hemorrhage. This study was to examine glycyrrhizin's effect on proinflammatory cytokines and TLRs in SAH rats. Administration with glycyrrhizin was initiated 24 hr before and 1 hr later using osmotic minipump. Basilar arteries were harvested to examine TLRs mRNA and protein (rt-PCR and western blot) and CSF cytokines (rt-PCR). Morphologically, deformed endothelium, tortuous elastic lamina, and smooth muscle necrosis were observed in the SAH rats, but were absent in the glycyrrhizin pretreatment group. The TLR-3 protein level was not increased in SAH animals, compared with the controls, while that of TLR-2 and -4 in the SAH only and SAH plus vehicle groups was significantly elevated ( $P < 0.01$ ). Pretreatment and treatment with glycyrrhizin reduced TLR-2 and -4 by  $28 \pm 8\%$  and  $33.4 \pm 9.2\%$ , respectively. Likewise, glycyrrhizin was able to reduce the IL-1 $\beta$  and MCP-1 mRNA levels. This study shows glycyrrhizin exerts anti-inflammatory effects on SAH induced vasospasm and attenuates the ultrashort time expression of TLRs, like TLR-2 and -4. It corresponds to SAH induced early brain injury. These findings offer credit to the antivasospastic effect of glycyrrhizin and its effect on SAH induced early brain injury.

## 1. Introduction

Subarachnoid hemorrhage- (SAH-) induced cerebral vasospasm remains a major cause of morbidity and mortality in patients suffering from aneurism rupture [1–3]. As effective therapies to change this condition are lacking, the precise mechanisms of this disease content require further investigation. A growing body of researches indicates that two hypotheses include the role of endothelins [4] and free nitric oxide [5] and another role of inflammation in the development and maintenance of delayed vasoconstriction [6–12]. If SAH occurs, a cascade of cellular and molecular events is elicited by the presence of oxyhemolysate in the subarachnoid space, and these culminate in a vigorous inflammatory response [9]. The putative importance of inflammatory activity has not been fully emphasized, even though its role in the genesis of cerebral vasospasm has been recognized.

Till now, various inflammatory constituents including adhesion molecules, cytokines, leukocytes, immunoglobulins, and complements have been observed in the pathogenesis of SAH-induced early brain injury and delayed cerebral vasospasm [1, 3, 4, 7–9].

The levels of cytokines have been found over time to increase in cerebrospinal fluid (CSF) after SAH in our previous study [13]. However, the benefits of inflammation development after SAH remain unclear. Toll-like receptors (TLRs) are a family of pattern recognition receptors and act as a signal-transducer for microbial peptidoglycan and lipoglycan, which initiate innate immunity [7, 11, 14–16]. TLR-2 is expressed on microglia, Schwann cells, monocytes, dendritic cells, polymorphonuclear leukocytes, pulmonary alveoli, and renal tubules [17, 18]; TLR-3 is abundant in placental, pancreatic, and dendritic cells; TLR-4 exists in placental cells and myelocytes [18]. Recent studies indicate

whether TLR-2 knock-out mice expressed severe neurological deterioration in the presence of  $\beta$ -amyloid deposit or not [15]. In Cole et al.'s study, TLR-3 showed a protective role in mouse models of atherosclerosis through activating interferon regulatory factor 3 and inducing type I interferons [17]. Likewise, through acting with endogenous ox-LDL, heat shock protein 60 and 70, fibrinogen, and fibronectin in the CSF or brain, TLR-4 has been demonstrated to be able to initiate inflammation related to stroke, Alzheimer's disease, Huntington's disease, and Parkinson's disease [19, 20]. A spinal cord injury study shows that both TLR-2 and -4 play a protective role through regulating inflammation, gliosis, and demyelination [21]. The novel interpretation illustrates that TLRs can lead to new exciting avenues for future investigation into the prevention and treatment of stroke and other neurological degenerative disorders. It may be reasonable to postulate that TLRs are involved in inflammation in the brain and play a putative role in early brain damage after SAH.

Glycyrrhizin, a traditional herbal remedy, has been used for the treatment of chronic virus hepatitis and gastric ulcer in Asia [22]. Recently, several studies have demonstrated that the chemical constituents of glycyrrhizin have a variety of biological effects such as anti-inflammatory, antioxidant activities and free radical scavenging activity, antiulcer, anti-hepatotoxic, antimicrobial, cytoprotective, cytotoxic activities (7Y11), and also neuroprotective effects against forebrain ischemic damage [22–26]. Moreover, glycyrrhizin's anti-inflammatory effects can be attributed to its inhibition of de novo TLRs. In Kim et al.'s study, glycyrrhizin was reported to be neuroprotective by binding the high-mobility group box 1 protein and reducing proinflammatory cytokine activities in an ischemic brain [27, 28]. In our pilot study, purified glycyrrhizin extract in 0.01% ammonium chloride solution made the compound easily dissolved in vehicle (0.9% sodium chloride) for transportation to the central nerve system and ready for further study.

Taking these findings together, we designed this study to evaluate the relationship between SAH-induced vasospasm and expressed levels of TLR-2, -3, and -4, which are believed to play a critical role in sterile inflammation and against SAH-induced ischemic brain injury and delayed vasoconstriction. The effects of glycyrrhizin on the prevention of cerebral vasospasm after SAH and alterations of TLRs mRNA were estimated.

## 2. Methods

**2.1. Materials.** Glycyrrhizin was further purified according to the modified protocol described by Cui et al. [14]. Glycyrrhizin extract was bought from JoryHerb Ltd., Shaanxi, China. The extract was inoculated with ammonium chloride for 2 hr and then let dry in methanol overnight in 24°C, so glycyrrhizin was obtained (purity 75 to 90% via chromatographic analysis). Monoclonal anti-rat interleukin 1- $\beta$  antibody was bought from Sigma-Aldrich (No 1010 Huai Hai Zhong Road, Shanghai 200031, PRC). Anti-rat interleukin 2, monoclonal anti-rat interleukin 6, and horseradish peroxidase-labeled goat anti-mouse IgG antibodies were obtained from Abcam (1 Kendall Square, Ste 341 Cambridge,

MA 02139-1517, USA), BD Transduction Lab (BD Biosciences 2350 Qume Drive San Jose, CA, USA), Upstate Biotech (10 old barn road lake placid, NY, USA), Santa Cruz Biotech (Santa Cruz Biotechnology Inc., 2145 Delaware Avenue Santa Cruz, CA 95060, USA), and Chemicon International (28820 Single Oak Drive Temecula, CA 92590, USA), respectively. CNM protein extraction kits were from Biochain (3517 Breakwater Avenue, Hayward, CA 94545, USA). Glycyrrhizin was purified by Ms. Wu SC (Kaohsiung Medical University Hospital, 100 Tzyou 1st Road, Kaohsiung 807, Taiwan, ROC) and further extracted by Sephadex LH-20 multiple column chromatography for four times. (Pharmacia Fine Chemicals, Piscataway, NJ, USA).

**2.2. Induction of Experimental SAH.** Forty-five male Sprague-Dawley rats, weighing between 350–450 g (BioLasco Taiwan Co., Ltd., authorized by Charles River Lab), were used in this study. All experimental protocols were approved and under supervision by the University of Kaohsiung Medicine Animal Research Committee. The rats were anesthetized by an intramuscular injection of a mixture of 0.9 mg/100 gm xylazine and 5.5 mg/100 gm KetaVed. 0.3 mL arterial blood was withdrawn and injected into the cisterna magna using a 30-gauge needle under a stereotactic apparatus (Stoelting, Wood Dale, IL, US). After the induction, animals were placed in ventral recumbent position for 15 minutes to allow ventral blood clot formation. Perfusion-fixation on the animals was then performed. After close monitoring for respiratory distress and giving mechanical ventilation if necessary, the animals were returned to the vivarium when fully awake. They were placed in a habitat with a 12 hr light-dark cycle and had access to food and water ad libitum.

**2.3. General Design of Experiments and Treatment Groups.** The rats were randomly divided into the following five groups (nine animals/group): (1) sham operated (no SAH); (2) SAH only; (3) SAH plus vehicle; (4) SAH plus glycyrrhizin (5 mg/kg/day); and (5) SAH plus precondition glycyrrhizin (5 mg/kg/day). The dosage was adjusted according to our pilot study. The first injections given to animals were administered 24 hr before (pretreatment) and 1 hr after induction of SAH (treatment) by using an osmotic mini-pump (Alzet corp, Palo Alto, Ca). After the animals received reanesthesia, CSF sampling was obtained through a 30-gauge needle into the cranial-cervical junction by using stereotactic apparatus (Stoelting, Wood Dale, IL, US). The animals were sacrificed by perfusion-fixation 72 hr after 2nd SAH. Cortical tissue samples were obtained by means of placing a 22-gauge needle inserted 5 mm in depth into the skull bone ( $N = 5$ ) through a burr hole craniectomy (2 mm apart from the bregma) at 24, 48, and 72 hrs.

**2.4. Perfusion-Fixation.** By the end of SAH, the animals were reanesthetized by administration of pancuronium bromide (0.3 mg/kg). The femoral artery was catheterized to monitor blood pressure and obtain blood samples to determine arterial blood gas, Na, K, GOT, and GPT levels. Perfusion-fixation was then performed. As the thorax was opened, the left

ventricle was cannulated with a NO16 catheter, the descending aorta was clamped, and the right atrium was opened. Perfusion with 100 mL of 70 mm Hg of 0.01 M phosphate buffer (pH 7.4) was begun, followed by fixation with 160 mL 2% paraformaldehyde in the PBS solution at 36°C under a perfusion pressure of 60 mm Hg. Thereafter, the brain was harvested and immersed in a fixative at 4°C overnight. Visual inspection showed that formed subarachnoid clots covered the basilar artery (BA) in all SAH animals.

**2.5. Tissue Embedding.** The middle third of each BA was dissected for analysis. The BA was rinsed repeatedly with 0.1 mol/L PBS (pH 7.4), fixed in 1% osmium tetroxide in PBS for 1 hr, and then washed with PBS at 24°C. The arterial segments were dehydrated in a 1:1 mixture of propylene oxide and placed in an epoxy resin overnight. The specimens were flat-embedded in 100% epoxy resin and allowed to polymerize at 60°C for 48 hrs. 0.25  $\mu\text{m}$  in thickness of the BAs was cross-sectioned on an Ultracut E ultramicrotome (Reichert) and stained with 0.5% toluidine blue for further analysis.

**2.6. Tissue Morphometric Study.** Five selected arterial sections of each animal were analyzed by an investigator blinded to the experiment assigned. Measurement of the BA cross-sectional area was made using an automatic computer-assisted morphometer (Image 1, Universal Imaging Corp.). A single value for each animal was obtained from an average of five cross-sections from a given animal.

**2.7. Quantification of mRNA Expression of IL-1 $\beta$ , IL-6, IL-8, and MCP-1.** The ABI PRISM 7900 System (Applied Biosystems, Foster City, USA) was applied to establish the expression of mRNA (rt-PCR), according to the manufacturer's instructions. The mRNAs for IL-1 $\beta$ , IL-6, IL-8, and MCP-1 expression were examined, while 18S was assigned as a housekeeping gene owing to its stable expression. In brief, each sample was launched into a TaqMan Human Cytokine Card that enclosed probes and primers for specific targets as well as the 18S ribosomal RNA control. This procedure was operated through an affixed filling reservoir and a vacuum loading process by using the ABI PRISM Card Filling Station.

Target mRNAs were standardized next to the reference gene (18S) and final data were expressed as a relative fold from baseline values. Comparative mRNA expression was set by the Livak and Schmittgen  $\Delta\text{CT}$  method. The results were analyzed only if there were at least a 5-fold increase in the mRNA levels compared with baseline in order to allow for data consistency.

**2.8. Assay of TLR-2, -3, and -4 Protein via Western Blots.** The frozen cerebral homogenates, lysed in 20 mM Tris, pH 7.6, which contained 0.2% SDS, 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.11 IU/mL aprotinin (Sigma-Aldrich, Inc., Taipei, Taiwan.), were centrifuged at 12,000 r.p.m. for 20 min at 4°C. 60  $\mu\text{g}$  per lane of the protein extracts was obtained on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis

(SDS-PAGE) and conveyed onto a nitrocellulose membrane. These membranes were then nurtured in a blocking buffer (5% skim milk in Tris-buffered saline containing Tween 20 (TBS-t) (50 mmol/L Tris, pH 7.5, 0.15 mmol/L NaCl, 0.05% Tween 20)) and incubated with anti-TLR-2, -3 and -4 protein in PBS + Tween 20 (PBST) (1:200) and  $\beta$ -actin (1:8,000), respectively, and left overnight at 4°C. Subsequently, the incubated membranes were then treated with secondary antibody conjugated with horseradish peroxidase (HRP) in TBS-t at room temperature for 1 hr. Anti- $\beta$ -actin antibody was used as a control. The protein levels were estimated by the Bradford method using the Sigma-Aldrich protein assay kit. The blots were developed via an enhanced Pierce chemiluminescence, a GS-700 digitally scanned and Molecular Analyst. Relative optical densities were obtained by comparison of the measured values with the mean values from the SAH groups. Relative optical densities were obtained by comparison of the measured values with the mean values from the SAH plus vehicle group. The blot proteins were visualized by enhanced chemiluminescence (ECL) Western blot detection reagents (Amersham Pharmacia, IL, USA). Developed films were digitized by means of an Epson Perfection 1260 scanner (Seiko Corp, Nagano, Japan). The TLRs expression levels were normalized to  $\beta$ -actin for at least three times.

**2.9. Detection of TLR-2 and -4 mRNA by Rt-PCR.** The levels of expressed TLR-2 and -4 mRNA were determined by TriPure RT-PCR Reagent (Roche Diagnostics Corp. IN, USA) according to the manufacturer's instructions. The PCR primer sequences were designed according to the TLR-2 and -4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene sequences reported in GenBank. TLR-2 (429 bp; forward: 5'-GAATTGCATCACCGGTCAGAA-3'; reverse: 5'-CCTCTGAGATTTGACGCTTTGTC-3'); TLR-4 (235 bp; forward: 5'-TTCAGAACTTCAGTGGCTGGA-TT-3'; reverse: 5'-CCATGCCTTGTCTTCAATTGTTT-3'); GAPDH (347 bp; forward: 5'-GGAGCCAAAAGGGTC-ATC-3'; reverse: 5'-CCAGTGAGTTTCCCGTTC-3'). By being incubated with Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT) (Promega, WI, USA), the TLR-2, -4, and GAPDH cDNA were amplified. The amplified cDNA fragments were detected by agarose gel electrophoresis swirl with 1  $\mu\text{L}$  of ethidium bromide. The intensity of the gene bands was quantified by using comet assay method. GAPDH mRNA was used as a housekeeping gene for multiplexing because of its stable expression under subarachnoid hemorrhage stimuli. The tissue of seven animals was employed for RT-PCR and three measurements were used per animal to get a mean value. All experiments were repeated three times.

**2.10. Statistical Analysis.** Data are expressed as the means  $\pm$  standard deviation. For group comparisons, an analysis of variance with the Bonferroni post hoc test was used. Difference, at a probability value less than 0.05, was considered significant.

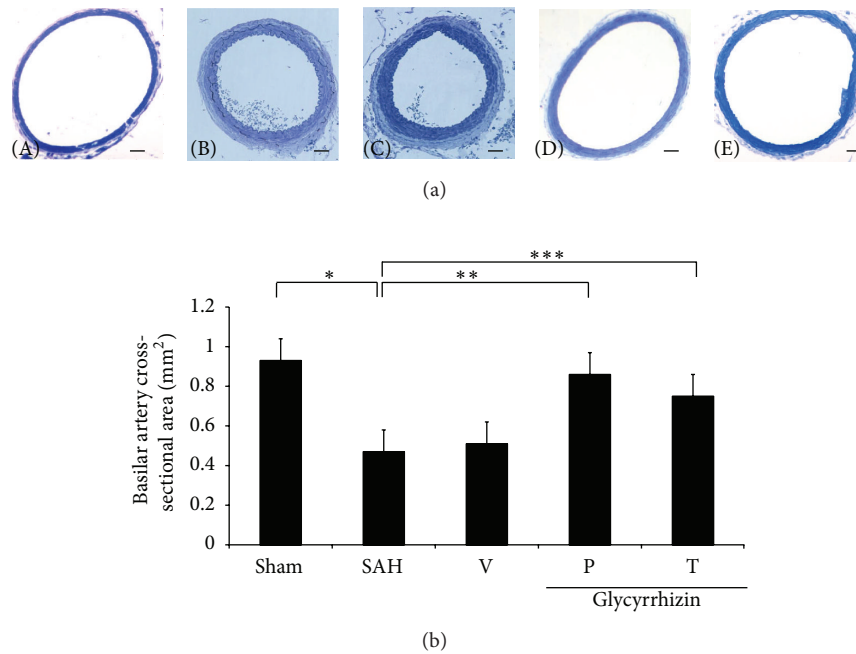


FIGURE 1: Comparison of lumen cross-sectional areas of the basilar artery. (a): representative micrographs of the cross-section of basilar artery obtained from the sham operated rat (A), the vehicle-treated SAH rat (B), SAH rat precondition treated with glycyrrhizin (C), and glycyrrhizin pretreatment in SAH animals (D), and SAH rat received glycyrrhizin after the induction of hemorrhage (E). Standard bar = 200  $\mu$ m (b): quantification of the lumen cross-sectional areas. All values are mean  $\pm$  SD ( $n = 9$ ). Glycyrrhizin exerted a potential to alleviate the vasospastic response when compared with the vehicle +SAH group. \*, \*\*, \*\*\*:  $P < 0.01$  compared with the SAH group.

### 3. Results

**3.1. General Observations.** Prior to decapitation, no significant differences were observed in the recorded physiological parameters, including body weight, body temperature, pH, blood pressure, and electrolytes among all groups. Morphologically, corrugated internal elastic layer (IEL), vacuolated endothelial cells in the BAs of the SAH only and SAH plus vehicle groups were exhibited. Vacuolated endothelial cells was less prominent in animals treated with glycyrrhizin. (Figure 1(a)) Vessels from healthy control animals and glycyrrhizin-pretreated animals had similar IELs.

**3.2. Cross Sectional Areas of BAs.** The mean cross-sectional areas of BAs in the SAH-only and SAH-plus vehicle groups were significantly reduced by 48% and 55%, respectively, when compared with the healthy controls (Figure 1(b)). Significant cerebral vasospasm did not develop in rats treated with glycyrrhizin, and its mean cross-sectional area was similar to that in the controls ( $P = 0.140$ ; Figure 1). The anti-vasospastic effect of glycyrrhizin achieved statistical significance when compared with the SAH-only or SAH-plus vehicle group ( $P < 0.01$ ).

**3.3. mRNA Expression of IL-1 $\beta$ , IL-6, IL-8, and MCP-1.** Subsequent to the induction of SAH, the serum concentrations of IL-1 $\beta$ , IL-6, IL-8, and MCP-1 were found to increase 1000- and 3000-fold at 72 hr, when compared with the sham operated group. Administration with glycyrrhizin significantly reduced cytokine levels by 8%, 16%, 37%, and 18% for IL-1 $\beta$ ,

IL-6, IL-8, and MCP-1, respectively, after SAH (Figure 2). The level of IL-6 was reduced in the glycyrrhizin treatment groups in a dose dependent mechanism in the pilot study. Both precondition and treatment with glycyrrhizin significantly reduced the levels of IL-1 $\beta$  and MCP-1 in animals subject to SAH. Meanwhile, treatment with glycyrrhizin tended to reduce the level of proinflammatory cytokines; the IL-6 and IL-8 levels failed to achieve statistical difference from the vehicle-treated +SAH group (Figure 2).

**3.4. Expression of TLR-2, -3, and -4 Proteins.** Western blot analysis showed increased TLR-2, -3, and -4 protein expression in the SAH rats ( $48 \pm 11\%$ ,  $13 \pm 8\%$  and  $51 \pm 9\%$ , resp.), when compared with the healthy controls. Treatment with glycyrrhizin significantly reduced the expression of TLR-2 and -4 protein in the animals subjected to SAH ( $P < 0.01$ ) to the levels of the controls (Figures 3, 4, and 5).

**3.5. Levels of TLR-2 and -4 mRNA.** TLR-2 and -4 mRNA were further evaluated with rt-PCR. Levels of TLR-2 and -4 mRNA were increased significantly in animals subjected to SAH (SAH-only and SAH-plus vehicle treatment groups) when compared with those in the healthy controls ( $P < 0.05$ , and  $P < 0.01$ , resp.). Treatment with glycyrrhizin decreased the levels of TLR-2 and -4 mRNA after SAH ( $P = 0.76$  and  $P < 0.05$ , resp.), while pretreatment with the same compound reduced the levels of TLR-2 and -4 mRNA to the normal controls when compared with that of SAH groups ( $P < 0.05$  and  $P < 0.01$ , resp.; Figures 6-7).

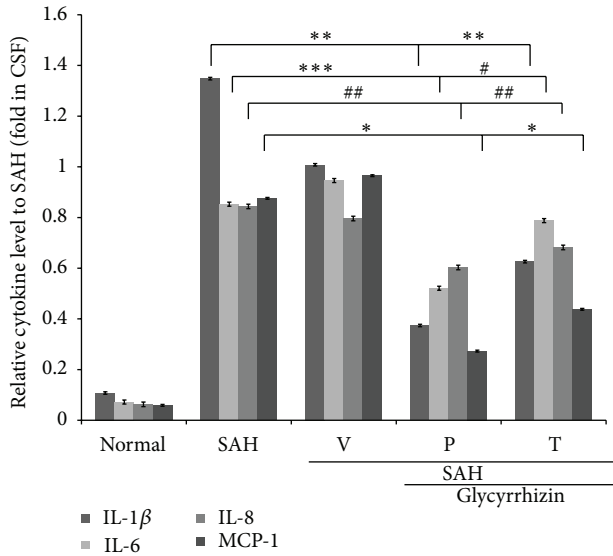


FIGURE 2: Effect of glycyrrhizin on proinflammatory cytokines in the CSF. IL-1 $\beta$ , IL-6, and MCP-1 were measured by rt-PCR. Samples were collected at 72 hr after the induction of SAH, when the vasoconstriction was robust. All values are mean  $\pm$  SD ( $n = 9$ ). \*, \*\*, \*\*\*:  $P < 0.01$ , and #, ##:  $P > 0.05$  when compared with the SAH group.

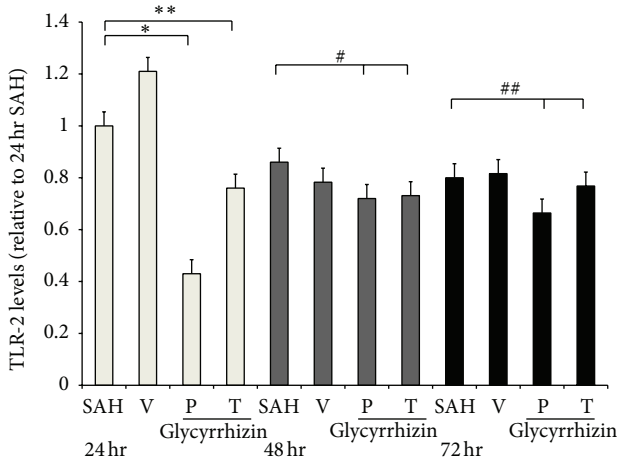


FIGURE 3: Expression of tissue Toll-like receptor 2 (TLR-2) on 24 hr, 48 hr, and 72 hr (Western blot). SAH; (V) SAH + vehicle; (P) Precondition glycyrrhizin +SAH; (T) glycyrrhizin +SAH. \*, \*\*:  $P < 0.01$ , animals in P and T groups compared with the SAH group. #, ##:  $P > 0.05$ , compared with the animals subject to SAH 48 hr and 72 hr, respectively.

#### 4. Discussion

Glycyrrhizin was proven able to attenuate the activation of TLRs subfamily in this SAH study. Activated TLR2, 3, and 4 associated with the release of inflammatory cytokines are important molecules that trigger an innate immune response. The inflammatory constituents produced by SAH were believed to mediate early brain injury and develop

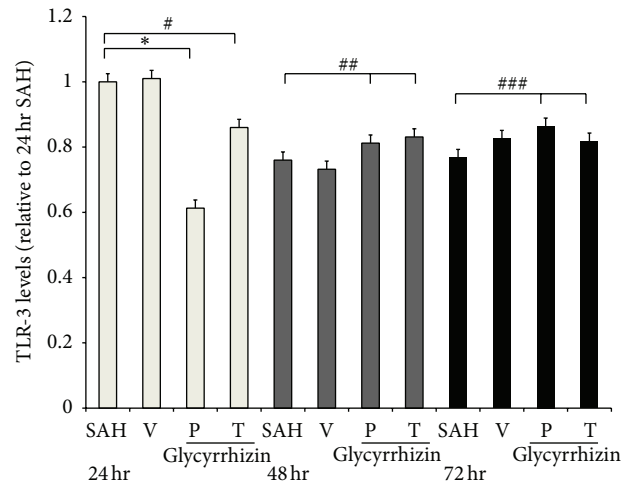


FIGURE 4: Toll-like receptor 3 (TLR-3) expression on 24, 48, and 72 hr, respectively (Western blot). All groups are identical to those shown in the legend of Figure 3. \*:  $P < 0.01$ ; #, ##, ###:  $P > 0.05$  represent glycyrrhizin treatment (24 hr), precondition and treatment with glycyrrhizin (48 and 72 hr, resp.), compared with the SAH groups.

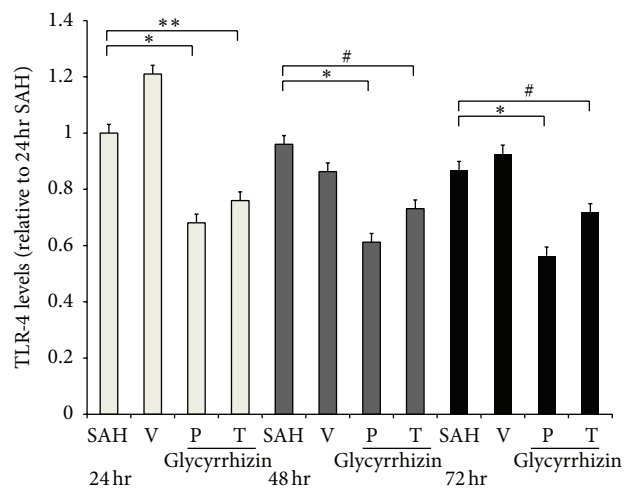


FIGURE 5: Time course expression of tissue Toll-like receptor 4 (TLR-4) (Western blot). \* $P < 0.01$ : precondition with glycyrrhizin (24, 48, and 72 hr, resp.) groups, and \*\*:  $P < 0.01$ , treatment with glycyrrhizin (24 hr) compared with the SAH groups. #:  $P > 0.05$  compared with the SAH group. All groups are equal to those publicized in Figure 3.

delayed vasoconstriction. Among the TLR family, only the TLR subfamily, especially TLR1 to 9, has the capacity to induce inflammatory signal transduction and elicit cerebral ischemic injury in rodents and humans [29, 30]. In a mammalian model, TLR1 was expressed in microglia and astrocytes; TLR2 was found to exist in oligodendrocytes and microglia, while TLR4 was expressed in the meninges, choroid plexus, and periventricle of the rat brain [30]. TLR2, 3, and 4 play a role in pathogen recognition in the central

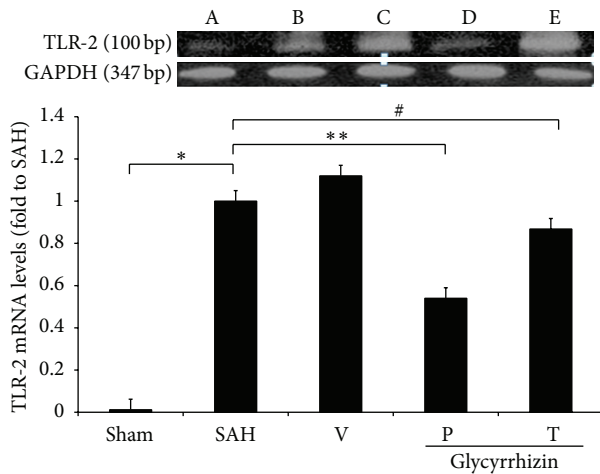


FIGURE 6: The activation TLR-2 mRNA among groups after induction of SAH. All groups are identical to those shown in the legend of Figure 1. All values are mean  $\pm$  SD ( $n = 9$ ). \*, \*\*,  $P < 0.01$ , and #:  $0.1 > P > 0.05$  compared with the SAH group.

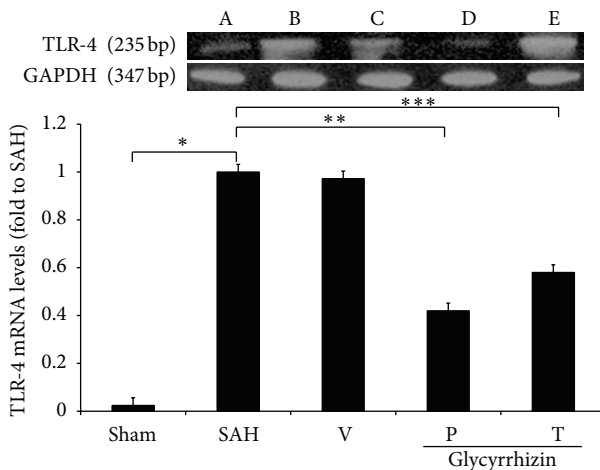


FIGURE 7: The alteration of TLR-4 mRNA in animals following SAH. All values are mean  $\pm$  SD ( $n = 9$ ). \*, \*\*, \*\*\*:  $P < 0.01$ , when compared with the SAH group. All groups are equal to those publicized in Figure 1.

nerve system by activation of both microglia-related neuroinflammation and dendritic cells and macrophage-related T-cell immunity. These molecules also mediate the production of cytokines, which are necessary for the development of T and B lymphocyte-related immune responses. In Smithason et al.'s study [31], LPS, a selective TLR4-receptor agonist, is responsible for the systemic inflammatory response-induced septic shock. Likewise, a TLR4 agonist is found to be able to worsen both vasospasm and cognitive behavior in mice as well as induce some Type-I interferon after stimulation with LPS. The study of TLR4 and TLR2 knockout mice revealed reduced infarct size was found after ischemic injury compared to the wild-type.

Our previous study revealed increased adhesion molecules and cytokine levels in cerebrospinal fluid (CSF)

after aneurysmal SAH. However, the relationships among the development of inflammatory response, early brain injury, and delayed cerebral ischemia in the brain after SAH need to be clarified. In Carr et al.'s study [13], the CSF inflammatory intermediaries increase after SAH, while the change in serum is not reliable. The upregulation of ET-1 peaks 3-4 days after SAH. Subsequently through negative feedback, activation of endothelial nitric oxide synthetase (eNOs) depletes NO, which plays a critical role in mediating vascular tone through a  $\text{Na}^+$ - $\text{K}^+$  channel and further results in vasodilation. Bowman et al. [32] also demonstrated that a polyclonal antibody targeted against IL-6 was able to alleviate SAH-induced vasoconstriction in a femoral artery study. The cumulative findings indicate that the upregulation of proinflammatory cytokines is antecedent to radiographic vasospasm (peak at 4th to 14th days after SAH), and attenuation of cytokines tends to minimize vascular constriction and reduced early brain ischemia in animals. Kim et al. [33] also declared monocyte chemoattractant protein-1 (MCP-1) is able to predict outcome and vasospasm following aneurysmal SAH. Elevation in the mRNA levels for IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ , and adhesion molecules has been observed in the pilot study [34].

TLRs are proven to be involved in a cascade of inflammation related stroke, Alzheimer's disease, Huntington's disease, and Parkinson's disease [35]. The activation of these pathways leads to the expression of various proinflammatory cytokines, chemokines such as TNF- $\alpha$ , IL-1 $\beta$  and regulated upon activation, normal T-cell expressed and secreted (RANTES), and the secretion of IFNs [20]. The induction of these responses is mainly triggered by the activation of NF- $\kappa$ B and MAPK pathways, a consequence common to the stimulation of all TLRs, which has been proven to induce neuron apoptosis. In this study, induced TLR4 mRNA was observed in the SAH groups as well as the expression of TLR4 protein. Treatment with glycyrrhizin significantly reduced TLR4 protein to the level of healthy control. However, TLR4 mRNA levels in the glycyrrhizin treatment group were lower than those in the SAH only and SAH plus vehicle groups and did not differ significantly from those in the control group. This finding implies that glycyrrhizin may attenuate SAH-induced vasospasm by decreasing immune-related TLR4 signaling.

Furthermore, in this study, upregulation of TLR2 protein and mRNA expressions were also significantly induced by experimental SAH. TLR2 mRNA was observed expressed in atherosclerosis-induced endothelial cell damage [15]. Unlike TLR4 which was observed in microglia in cerebral ischemia [36], activated TLR2 was short-lived and believed primary to the activation of TLR4, and both were induced by various inflammasome in the episode of SAH. An on-growing body of evidence indicates that stress on the central nervous system triggers an innate immune intervention in order to respond to risky stimuli. Endothelium and smooth muscle of the cerebral blood vessels express TLRs and can respond to stimulation [29, 30]. TLR4, prominently observed in the endothelial cells of the capillaries, are found to trigger the endothelium dysfunction and vascular inflammation following experimental SAH [11, 36]. A series of neuro- and perivascular inflammations would lead to the disruption or

dysfunction of the blood-brain barrier (BBB), which ends in profound cerebral reperfusion injury and early brain damage [11]. Conclusively, continuous release minipump aids to the immunosuppressant effect of glycyrrhizin on the upregulation of TLR2, 3, and 4. Significant attenuation of TLR2, and 4 mRNA in the glycyrrhizin groups corresponds to the level of TLR2 and 4 as well as the change of proinflammatory cytokines in animals subject to SAH. These findings also provide evidence that neuroinflammation mediates SAH-induced vasospasm and also suggests that glycyrrhizin could play a role in therapeutics in future studies. Further study of glycyrrhizin will focus on the MARK pathway and SAH-induced early brain injury.

## Abbreviations

BA:	Basilar artery
CSF:	Cerebrospinal fluid
DIND:	Delayed ischemic neurological deficit
ET:	Endothelin
eNOS:	Endothelial nitric oxide synthase
HMGB-1:	High-mobility group box 1
HRP:	Horseradish peroxidase
IEL:	Internal elastic lamina
IL-1 $\beta$ :	Interleukin-1 $\beta$
IL-6:	Interleukin-6
IL-8:	Interleukin-8
MAPK:	Mitogen-activated protein kinase
MCP-1:	Monocyte chemoattractant protein-1
LCA:	Leukocyte common antigen
PBS:	Phosphate-buffered saline
SAH:	Subarachnoid hemorrhage
PDGF:	Platelet derived growth factor
RANTES:	Regulated upon activation, normal T-cell expressed and secreted
TGF- $\beta$ 1:	Transforming growth factor- $\beta$ 1
TLRs:	Toll-like receptors
VEGF:	Vascular endothelial growth factor.

## Conflict of Interests

The authors disclaim any conflict of interests related to this paper.

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