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Glycyrrhiza uralensis Fisch. and its active components mitigate Semen Strychni-induced neurotoxicity through regulating high mobility group box 1 (HMGB1) translocation

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ABSTRACT

Semen Strychni has long been used for the treatment of rheumatoid arthritis, facioplegia and myasthenia gravis due to its anti-inflammation and anti-nociceptive properties in China. However, the fatal neurotoxicity of *Semen Strychni* has limited its wider clinical application. To investigate the acute toxicity induced by *Semen Strychni* and the detoxification of liquorice, we evaluated inflammation, oxidative stress and the translocation of high mobility group box 1 (HMGB1) in rats. As a result, there were obvious oxidative stress and inflammation in hippocampus after the *Semen Strychni* extracts (STR) treatment in rats. Liquorice extracts (LE) and its three active monomers – glycyrrhizic acid (GA), liquiritigenin (LIQ), isoliquiritigenin (ISL) showed the potential for mitigating STR-induced neurotoxicity. HMGB1 levels in cytoplasm and serum and the levels of two downstream receptors RAGE and TLR4 were significantly increased after STR treatment. Through using LE and the monomers, the nucleocytoplasmic transport and release of HMGB1 were inhibited. In addition, the binding between HMGB1 and TLR4 was weakened in detoxification groups comparing with the STR group. Taken together, these findings indicated that liquorice and its active components alleviated acute neurotoxicity induced by *Semen Strychni* partly via HMGB1-related pathway.

1. Introduction

Semen Strychni is obtained from the dried mature seeds of the medical plant *Strychnos Nux Vomica*, which contains a lot of alkaloids including strychnine and brucine. As a traditional Chinese medicine, it can be used for treating rheumatoid arthritis and facial nerve paralysis according to the Chinese Pharmacopoeia. Modern pharmacological studies have demonstrated that Strychnos alkaloids (SAs) could exert anti-angiogenesis, anti-nociceptive and anti-tumor effects [1,2,29]. However, strychnine and brucine are both bio-active and noxious ingredients of *Semen Strychni*. *Semen Strychni* has a narrow therapeutic window, which exerts great limitation to its clinical use [4,28]. When used at relatively high doses, it can cause epileptic-like symptoms which may lead to severe neurotoxicity and even death. According to recent researches, there would be serious oxidative stress and inflammation in brains of SAs poisoned rats, and the energy metabolism and endogenous substances would change as well [10,18,34,35]. Hence it is vital to clarify the neurotoxic mechanism of *Semen Strychni* and find an effective antidote to it.

Recently, it has been suggested that High mobility group box 1 (HMGB1) serves to induce neuroinflammation after brain injury including but not limited to epilepsy [7,32]. HMGB1 is a non-histone DNA-binding protein, which can stabilize nucleosome structure and promote gene transcription. As one of classical damage associated

Abbreviations: HMGB1, high mobility group box 1; SSE, *Semen Strychni* extracts; LE, liquorice extracts; GA, glycyrrhizic acid; LIQ, liquiritigenin; ISL, isoliquiritigenin; SAs, Strychnos alkaloids; DAMP, damage associated molecular patterns; TLRs, Toll-like Receptors; RAGE, receptor for advanced glycation end products; SIRT1, Sirtuin1; MAPK, mitogen-activated protein kinase; HO-1, Heme Oxygenase-1.

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molecular patterns (DAMPs), HMGB1 displays a high propensity to be a neuroinflammatory factor when released from the necrotic cells or stressed cells during the pathological process of brain injuries [7,12]. It is suggested that HMGB1 is a key initiator of neuroinflammation, which further triggers microglial activation and enhances neuroinflammation, partially by interacting with Toll-like Receptors (TLRs) and receptor for advanced glycation end products (RAGE) [14,20,26]. It is obvious that the function of HMGB1 depends on its cellular location, the translocation of HMGB1 from nucleus to cytoplasm and release in extracellular milieu is a key step in its DAMP functions. In the seizure mouse model, HMGB1 level was up-regulated and there was an accumulation in the serum [23]. Acute and massive neuronal death is inevitable after SAs administration, hence HMGB1 may be a key inflammatory cytokine in the continuous brain damage induced by SAs ([34,35]).

Glycyrrhiza uralensis Fisch. is a natural product which is used to relieve pain and reconcile various medicines according to traditional Chinese medicine theory [23,41]. The active components in liquorice contain triterpenoid such as glycyrrhizic acid (GA) and flavonoids including liquiritigenin (LIQ) and isoliquiritigenin (ISL), which exerts various pharmacological activities on neuroprotection, including anti-oxidant and anti-inflammation [3,46]. Emerging research showed that GA exerted neuroprotective efficacy through ameliorating brain injury and neurological deficits via blocking the HMGB1-TLR4 signaling in microglia-triggered inflammation as well as postischemic brain [41,55]. LIQ has been reported to prevent glutamate-induced hippocampal neuronal cell death by suppressing ROS overproduction and attenuation of MAPK activation [49]. Similarly, ISL was discovered to alleviate nerve conduction and nerve blood flow deficits in diabetic rats and thus showed neuroprotective effects partially by activating SIRT1, which could mediate the deacetylation of HMGB1 [30,52]. Effects on SIRT1 by GA have also been reported to protect renal tubular epithelial cell injury [11].The significance of SIRT1-mediated deacetylation of HMGB1 has been demonstrated in neuroinflammation-related diseases such as experimental traumatic brain injury model, neonatal hypoxic-ischemic brain injury as well as cognitive impairment [19]. Our previous study has found that the toxicity of brucine was significantly decreased after using liquorice [56]. Therefore, it is worthwhile to explore whether liquorice and its active components could mitigate severe neurotoxicity induced by *Semen Strychni* and the possible mechanisms.

In this study, a rat model of neurotoxicity induced by *Semen Strychni* was established, we assessed inflammation, oxidative stress and HMGB1-related signaling pathways in rats to evaluate the neuroprotective effects of the liquorice water extracts (LWE) and three main active constituents in liquorice – GA, LIQ and ISL. We also explored whether HMGB1 exerts as a DAMP in *Semen Strychni* poisoning. This project may contribute to the possible mechanism of liquorice detoxification effects on *Semen Strychni* in neural aspects, and may provide promising rescue of poisoned patients and improve the safety of clinical application of *Semen Strychni*.

2. Materials and methods

2.1. Chemicals, reagents and materials

Semen Strychni were purchased from Shenghaitang Herb Pieces Company (Anhui, China). Liquorice was purchased from Sanxiang Herb Pieces Company (Hunan, China). Glycyrrhizin, liquiritigenin and isoliquiritigenin were obtained from Yuancheng Technology Co.Ltd (Hubei, China). Antibodies against HMGB1 (10829–1-AP), TLR4 (19811–1-AP), HO-1 (66743–1-Ig) and Bax (50599–2-Ig) were obtained from Proteintech (Chicago, USA). Antibody against acetylated lysine (HW098) was obtained from Signalway Antibody (Maryland, USA). RAGE (AF5309) and SIRT1 (DF6033) antibodies were from Affintity Biosciences (Jiangsu, China). RIPA lysis buffer, Cell lysis buffer for Western and IP (WIP), BCA protein assay kit and lipid peroxidation MDA assay kit were obtained from Beyotime (Shanghai, China). Nucleus and cytoplasmic extraction kit was obtained from Bestbio (Shanghai, China). The ELISA kit for neuron-specific enolase (NSE), nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) were purchased from Cusabio (Wuhan, China). The TNF-α ELISA kit was obtained from Thermo Fisher Scientific (Waltham, USA) and IL-6 ELISA kit was obtained from Mluti sciences (Hangzhou, China). The terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) kit was obtained from Roche (Indianapolis, USA).

2.2. Herb extraction

The smashed raw *Semen Strychni* was extracted by refluxing with 75% acidic ethanol (pH=5, 1:12, w/v) three times and 1 h for each time. The collected filtrate was concentrated under reduced pressure until there was no ethanol in the extraction. Then filter through a 0.45 μm microporous membrane. Adjust the extraction pH at 6.5 with 1 mol/L NaOH solution. Then made the final extraction concentration at 0.05 g raw *Semen Strychni*/0.5% CMC-Na solution ml using 1% CMC-Na solution and water. The composition of the Semen Strychni extract was detected by high-performance liquid chromatography coupled with tandem mass spectrometry and ultraviolet detector (HPLC-UV-MS) (Supplementary Material).

Raw liquorice soaked for 12 h in water (1:5, w/v) before extraction. Then extracted by refluxing two times and 2 h for each time. The collected filtrate was concentrated. Then made the final extraction concentration at 1.2 g raw liquorice/0.5% CMC-Na solution ml using 1% CMC-Na solution and water. Through the HPLC method we established before, the concentration of GA, LIQ and ISL in the LWE were 11.89, 0.58 and 0.17 mg/ml respectively [56]. The concentration of brucine and strychni in the STR were 0.305 mg/ml and 0.126 mg.ml respectively.

2.3. Animal and experiment design

Animal experiments were carried out according to institutional guidelines for the care and use of laboratory animals, and approved by the Animal Ethics Committee of Central South University (approval ID: 2019sydw0022). The department of laboratory animals, Central South University (Changsha, China) provided male Sprague Dawley rats (200–300 g) in SPF grade and standardized animal facility (humidity,50 ± 10%; temperature, 22 ± 2 °C; room light, 12 h light/dark cycle). During the experiment, rats had free access to drinking water and standard rat chow.

At first, a pre-experiment was conducted to find the most appropriate dose of *Semen Strychni* extract (STR) that is tolerable and can cause obvious toxic effects. Rats were kept for a week after being purchased to adapt to the new environment. Then 42 rats were randomly divided into 6 groups (7 rats per group): control group, STR group, LWE group, GA group, LIQ group and ISL group. In this study, the rats received LWE or active components immediately after treated with STR every day for 7 days. The specific dosing protocols are as follows:

Control group: Rats were injected (i.p.) with vehicle (0.5% CMC-Na solution, 4 ml/kg), then orally administrated the vehicle (5 ml/kg) immediately.

STR group: Rats were injected (i.p.) with 0.2 g/kg/day *Semen Strychni* extract, then orally administrated the vehicle (5 ml/kg) immediately.

LWE group: Rats were injected (i.p.) with 0.2 g/kg/day *Semen Strychni* extract, then orally administrated the 6 g/kg/day LWE (5 ml/kg) immediately.

GA/LIQ/ISL group: Rats were injected (i.p.) with 0.2 g/kg/day *Semen Strychni* extract, then orally administrated the 50 mg/kg/day GA/LIQ/ISL solution (5 ml/kg) immediately.

2.4. Beam walking test

Motor coordination was assessed by the beam walking test [13,18,39]. 30 min after the drug administration, rats had to cross a 2 cm-wide beam, 80 cm length, elevated at 20 cm above the floor every day. In this experiment, we recorded the time that the rats crossed the beam every day using a timer. Rats were trained to adapt to walking on the beam one week before the experiment.

2.5. Sample collection

24 h after the last administration, rats were anesthetized with 1.5% avertin (10 ml/kg). Blood sample (approx. 4 ml) were collected from heart and transferred to anticoagulant tubes. Serum samples for ELISA and western blot were separated by centrifugation at 5000 rpm for 10 min at 4 °C. Then the serum samples were stored at − 70 °C until analysis.

Then rats were sacrificed after blood collection. The brain tissue was collected and washed with ice-cold PBS. Then the hippocampus was separated on an ice tray. All hippocampus samples were snap-frozen in liquid nitrogen and stored at − 70 °C until use.

2.6. Histopathology

At the end of the experiment, one rat of each group was transcardially perfused with normal saline followed by 4% paraformaldehyde. Brains were removed and fixed in 4% paraformaldehyde for 24 h. Brain tissue was paraffin-embedded and sliced into sections of 4 μm thick for staining.

For morphological observation, the brain sections were dewaxed in xylene, rehydrated through decreasing concentrations of ethanol, finally washed in PBS. And then stained with hematoxylin and eosin (H&E). After staining, sections were dehydrated through increasing concentrations of ethanol and xylene.

Apoptotic cells were detected using a TUNEL kit according to the manufacturer's instructions. Brain slices were incubated with NeuN (1:100) overnight at 4 °C, and after washing in PBS for three times, the slices were incubated with TUNEL reaction mixture for 1 h at 37 °C. The slices were scanned and photographed using a microscope.

The distribution of HMGB1 in the brain tissue were observed through immunohistochemical staining. The brain sections were deparaffinized with xylene and rehydrated in a graded series of alcohol. Antigen retrieval was carried out by microwaving in citric acid buffer. Then the brain sections were incubated with HMGB1 primary antibody (1:500) at 4 °C overnight. Sections were rinsed and incubated in the appropriate secondary antibody for 1 h at room temperature. DAB was used after secondary antibody. Sections were then dehydrated using an alcohol gradient, cleared in xylene and mounted for microscopic observation.

2.7. Oxidative stress marker assessment

Hippocampus tissues were homogenized in WIP lysis buffer (1:10, w/v). The homogenates were centrifuged at 12,000 g for 10 min, 4 °C. The protein concentration of supernatant was quantified using the BCA kit. Subsequent experimental steps to determine malondialdehyde (MDA) were performed according to the kit manufacturer's instructions.

2.8. Enzyme-linked immunosorbent assay (ELISA)

Hippocampus tissue was homogenized with PBS (1:10, w/v) and stored at − 20 °C overnight. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 min at 5000 g, 4 °C. The supernatant was removed and assayed immediately. Inflammatory factors in hippocampus tissue were detected using ELISA kit for TNF-α and IL-6. In addition, NSE, NGF and BDNF levels in hippocampus were all detected by ELISA. The procedures were all followed by the manufacturer's instructions.

2.9. Western blot analysis

Brain hippocampus tissues were centrifuged at 12,000 g for 10 min following homogenization in RIPA lysis buffer (1:10, w/v). The supernatants were collected. The whole protein samples were obtained by this method. The nucleus and cytoplasm protein were obtained by using the nucleus and cytoplasmic extraction kit. All protein samples were determined by BCA protein assay kit. Make appropriate dilution of serum and tissue samples based on the protein concentration. Then the samples were mixed with 5× loading buffer, then were boiled at 100 °C for 5 min and stored at − 70 °C until further analysis.

20 μg of protein from brain hippocampus protein was resolved on 10% SDS-PAGE gel electrophoresis. After electrophoresis, gels were transferred onto PVDF membranes using wet transfer at 252 mA for 90 min, 4 °C. The membranes were blocked for 1.5 h in TBST-buffered saline containing 5% (w/v) skim milk. The membranes were incubated with HMGB1, HO-1, SIRT1, Bax, TLR4, RAGE, acetylated-lysine, albumin and GAPDH primary antibody at 1:2000, 1:5000, 1:6000, 1:800, 1:6000, 1:800, 1:2500 and 1:3000 dilutions overnight at 4 °C, respectively. Then, the membranes were incubated with anti-rabbit or anti-mouse HRP-conjugated secondary antibody for 60 min, respectively. Protein bands were detected using chemiluminescence detection reagents. Band intensity was quantified by scanning densitometry. Each measurement was made at least three times.

2.10. Co-immunoprecipitation

Hippocampus tissues were centrifuged at 12,000 g for 10 min following homogenization in WIP lysis buffer (1:20, w/v). The supernatants were collected. Adjust the protein concentration at about 5 μg/ml with PBS. 200 μl tissue extract proteins were incubated with 2 μg HMGB1 antibody overnight to enable a protein-antibody interaction in solution, followed by 2 h incubation with protein A/G-coupled agarose beads. The beads were then washed 3 times using washing buffer. Then the isolated protein-bead conjugates were diluted with 1 × loading buffer and were boiled at 100 °C for 5 min. After centrifugation (13,000 g, 10 min, room temperature), the supernatants were collected and used for Western blot analysis. The Western blot was conducted using RAGE or TLR4 antibody to determine the relationship between HMGB1 and the receptor. In addition, using lysine-acetylation antibody to measure the acetylation of HMGB1.

2.11. Statistical analysis

Results were statistically expressed as mean ± standard deviation with SPSS 18.0. Single factor analysis of variance was used to compared the groups with homogeneous variance, and LSD-t test was used in pairwise comparison. $P < 0.05$ indicated statistical significance, While $P < 0.01$ indicated that the difference was very significant.

3. Results

3.1. Liquorice treatment ameliorated Semen Strychni-induced injury

During the one-week injection cycle, there was an increased level in LWE group as well as liquorice active components groups (GA, LIQ, ISL group) compared with STR group (Fig. 1) in motor coordination assessment. Along with the process of the beam walking test, the time for rats to cross the bean became shorter. Compared with the control group, it took more time for rats to cross the beam in the STR group ($p < 0.01$), while LWE administration significantly eliminated this rise in the five of seven days of the test. In monomer detoxification groups, GA, LIQ and ISL all improved the motor coordination of rats, of which GA played a relatively strong effect. In addition, H&E staining of rat hippocampus

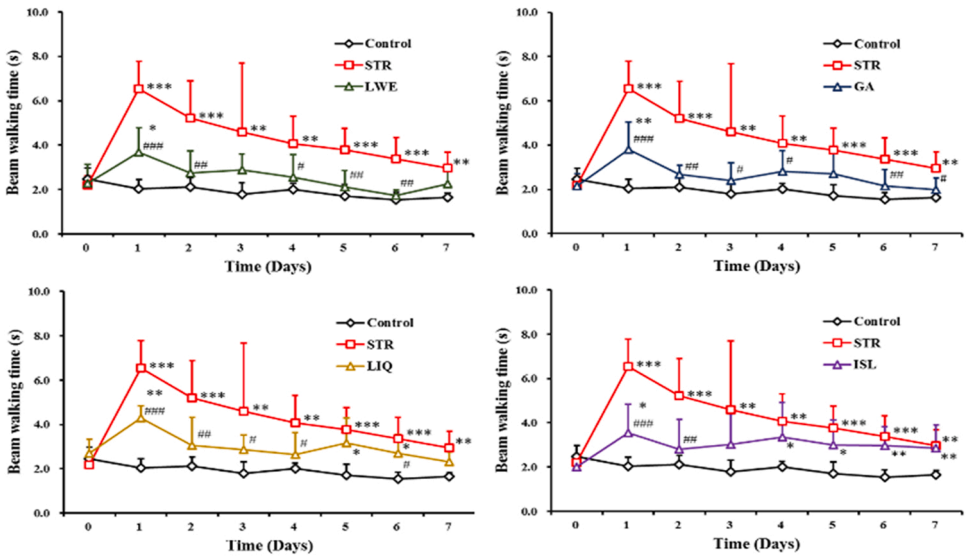


Fig. 1. The beam walking time comparison between the control group, the STR group and four detoxification groups (LWE, GA, LIQ, ISL). Data were presented as mean ± SD (n = 6); *p < 0.05, **p < 0.01, ***p < 0.001 vs. the control group and #p < 0.05, ##p < 0.01, ###p < 0.001.

was performed to evaluate the neuropathological injury, which can be characterized by neuronal morphology, liquefactive necrosis and neuronophagia. There were obvious liquefactive necrosis and karyopyknosis of neurons in the hippocampus in the STR group but not in the LWE and monomer detoxification groups (Fig. 2). Both motor coordination and histopathological examination showed that administration of LWE could attenuate *Semen Strychni* induced injury in rats. Among the three monomers of liquorice, the effect of liquiritigenin was relatively weak.

3.2. Liquorice treatment attenuated cell apoptosis and oxidative stress in brains of Semen Strychni-induced rats

According to the TUNEL staining (Fig. 3), oral administration of *Semen Strychni* extract caused obvious cell apoptosis in the hippocampus. Meanwhile, the control, LWE and active components groups showed a very low apoptotic fraction of neurons. Furthermore, treatment with LWE and monomers could also reduce the level of Bax, a pro-apoptotic factor ($p < 0.05$). However, the level of Bax was significantly increased in the STR group ($p < 0.05$) (Fig. 4A).

Brain tissue is rich in lipids. When overexposed to *Semen Strychni,* symptoms such as convulsions and difficulty in breathing could cause a dramatic increase in oxygen consumption in the brain and lead to oxidative stress. So as a product of lipid oxidation in organisms, malondialdehyde (MDA) can be used as a biomarker for evaluating oxidative stress. As seen in Fig. 4B, the MDA content was increased in the STR group by 55.4% when compared to the control group ($p < 0.05$), and LWE treatment significantly decreased MDA level. Among the monomer groups, ISL showed the strongest effect on reducing oxidative stress and neuronal apoptosis induced by *Semen Strychni.*

3.3. Liquorice reduced the level of pro-inflammatory cytokines and lessened neurofunctional disturbance after Semen Strychni poisoning

Emerging evidence suggests that neuroinflammation plays a key role in central nervous system (CNS) injury, and could be detrimental to neuronal cells [50]. Pro-inflammatory cytokines such as TNF-α and IL-6 could be induced by the activation effect of HMGB1 on family members of mitogen-activated protein kinase (MAPK) during sustain damage [7]. Figs. 4C and 5A showed that the TNF-α and IL-6 levels in serum were greatly increased in the STR group as compared with the control group,

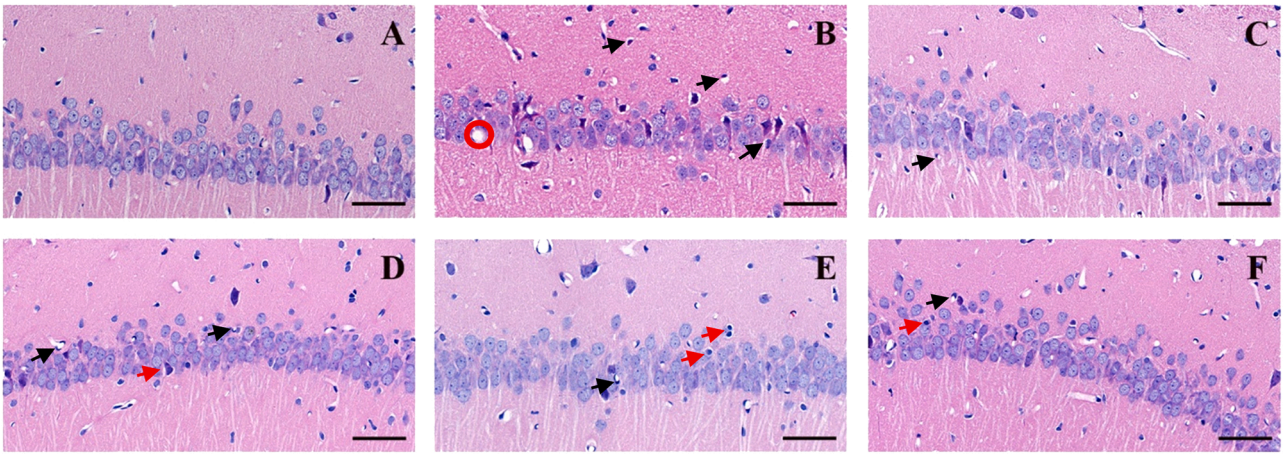


Fig. 2. Effects of Semen Strychni, liquorice and its active components on histological changes in hippocampus. Representative histopathological photographs of rat hippocampus (400×) in control (A), STR (B), LWE (C), GA(D), LIQ (E) and ISL (F) group. STR: rats treated with 0.2 g/kg/day STR, LWE: rats treated with 0.2 g/kg/day STR and 6 g/kg/day LWE, GA/LIQ/ISL are rats treated with 0.2 g/kg/day STR and 50 mg/kg/day GA/LIQ/ISL solution respectively. Red circle, liquefactive necrosis; black arrow, gitter cell; red arrow, neuronophagia. Scale bar = 70 μm.

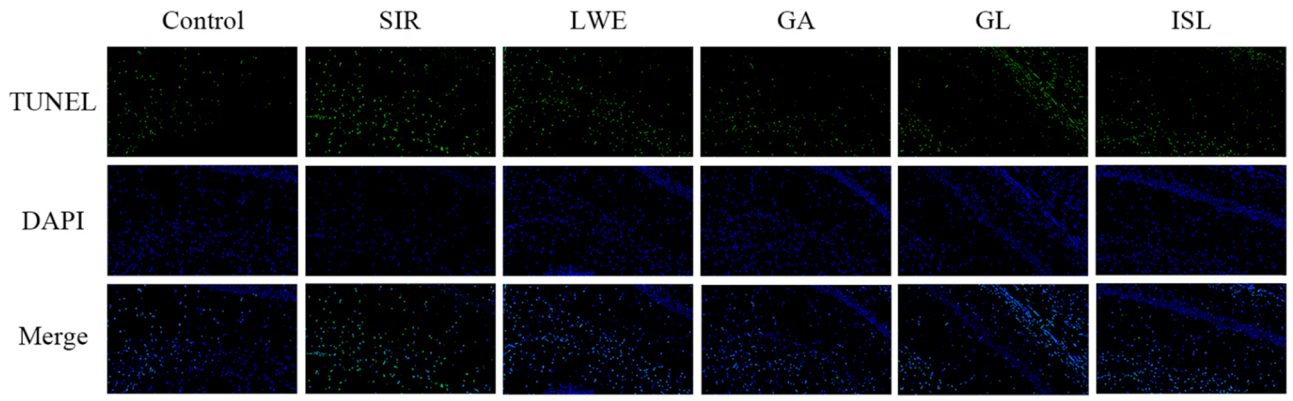


Fig. 3. Representative photographs of terminal deoxynucleotidy transferase-meditated deoxyuridine 5-triphosphate-biotin end labeling (TUNEL) staining. Green fluorescence shows TUNEL-positive nuclei; blue fluorescence shows nuclei of total hippocampus. Original magnification ×5. STR: rats treated with 0.2 g/kg/day STR, LWE: rats treated with 0.2 g/kg/day STR and 6 g/kg/day LWE, GA/LIQ/ISL are rats treated with 0.2 g/kg/day STR and 50 mg/kg/day GA/LIQ/ISL solution respectively.

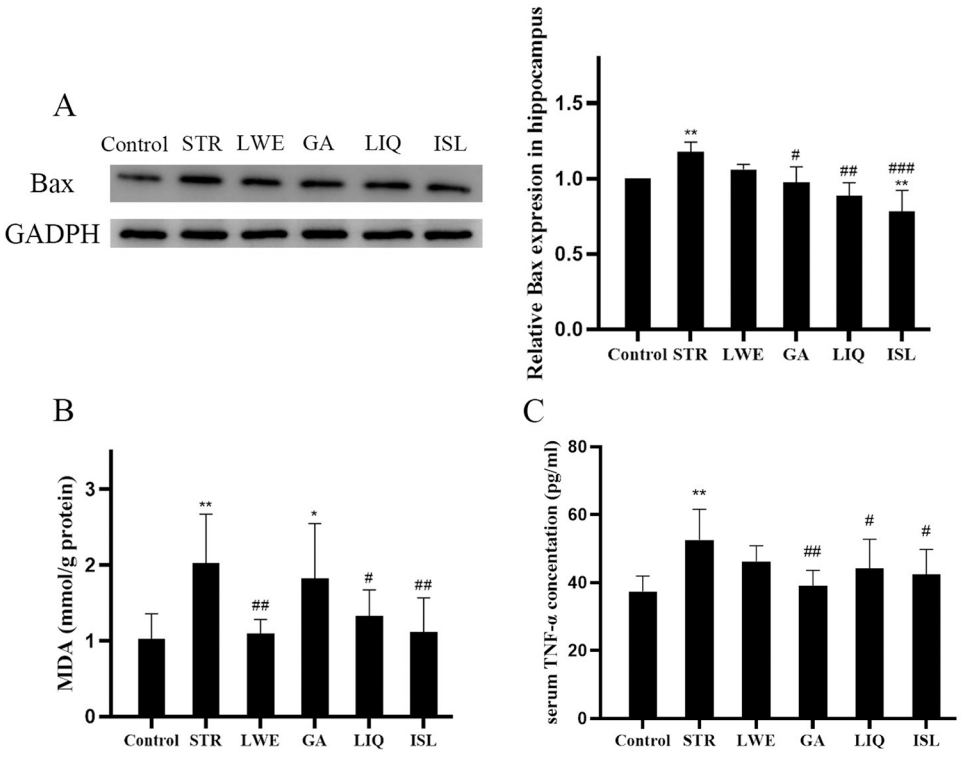


Fig. 4. The effect of liquorice treatment on Bax, MDA and TNF-α in S.Strychni induced rats. (A) Representative immunoblot and relative densitometric analysis of Bax expression in rat hippocampus from the control group, the STR group and four detoxification groups (LWE, GA, LIQ, ISL). (B) The determination results of MDA in hippocampus. (C) The determination results of TNF-α in hippocampus. Data represent the ratio of Bax and GAPDH and are shown as mean ± SD (n = 3 for A, n = 6 for B and C). *p < 0.05 vs. the control group and #p < 0.05, ##p < 0.01 vs. the STR group.

according to the calculation, they were increased by 144.4% ($p < 0.05$) and 40.5% ($p < 0.01$), respectively. Compared with the STR group, the rising trend of TNF-α and IL-6 in LWE group had not been reduced in a significant manner. Both LIQ and ISL alleviated the increase of the two inflammatory factors to a certain extent ($p < 0.05$). GA showed the strongest effect on decreasing TNF-α level ($p < 0.01$), but it had no significant effect on the reduction of IL-6.

NSE is a specific and sensitive biomarker of neuronal damage. According to the ELISA result (Fig. 5B), there was a robust increase in serum concentration of NSE in the STR group compared with the control group ($p < 0.01$), and treatment with liquorice and monomers reduced this effect significantly ($p < 0.05$). Furthermore, we evaluated the NGF and BDNF content in hippocampus. As shown in Figs. 5C and 5D, liquorice exerts a good neuroprotective effect by upregulating the two neurotrophic factors ($p < 0.05$). All of the three active components of liquorice caused a significant increase in NGF and BDNF levels compared with the STR group ($p < 0.001$).

3.4. *Liquorice mitigated nucleus-to-cytoplasm translocation and the circulating level of HMGB1 in Semen Strychni poisoning rats*

As a downstream mediator of systematic inflammation, HMGB1 is considered to play an important role in brain damage [19]. Through immunohistochemical staining, we explored the approximate HMGB1 distribution in the hippocampus after drug delivery cycle. From the immunohistochemical staining pictures (Fig. 6A), HMGB1 was mostly expressed in the nucleus in the control group. However, the nucleus-to-cytoplasm translocation was obviously increased in the STR group. HMGB1 was seriously diffused in the hippocampus tissue, which indicated that *Semen Strychni* poisoning had caused intense neuroinflammation. LWE, GA, LIQ and ISL all alleviated the efflux of HMGB1. In addition, in the LIQ and ISL groups, it seemed that the HMGB1 level

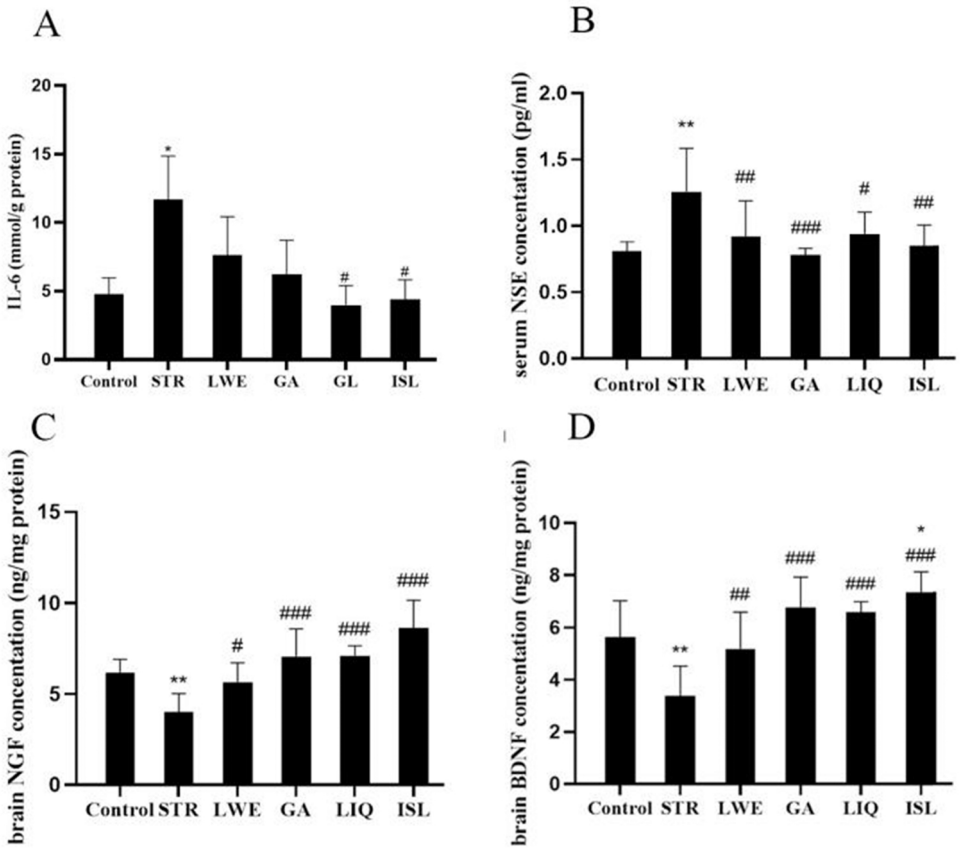


Fig. 5. Liquorice reduced inflammation and neuronal injury induced by Semen Strychni. The determination results of IL-6 (A) and NSE (B) in serum, NGF (C) and BDNF (D) in hippocampus. Data were presented as means ± SD for B, C, D or mean ± SEM for A (n = 6). *p < 0.05, **p < 0.01 vs. the control group and #p < 0.05, ##p < 0.01 vs. the STR group.

was decreased in total.

Western blot analysis was used to further clarify the distribution of HMGB1(Fig. 6B). From the data, it could be seen that in the STR group, HMGB1 was accumulated in the cytoplasm and further released to the serum ($p < 0.01$). Consistent with the immuno-histochemical result, LWE, LIQ and ISL all reduced the HMGB1 content in serum ($p < 0.05$). Moreover, the nucleus-to-cytoplasm translocation of HMGB1 induced by *Semen Strychni* extract was also inhibited by LWE and the active components. GA could inhibit the translocation of HMGB1 effectively ($p < 0.05$). In the meantime, LIQ and ISL both inhibited the nucleocytoplasmic transport as well as the release of HMGB1. The HMGB1 serum content in these two groups even fell below the control group level ($p < 0.01$).

3.5. Liquorice regulates HMGB1 nucleocytoplasmic transfer and releases partly by affecting the levels of HO-1 and deacetylase SIRT1

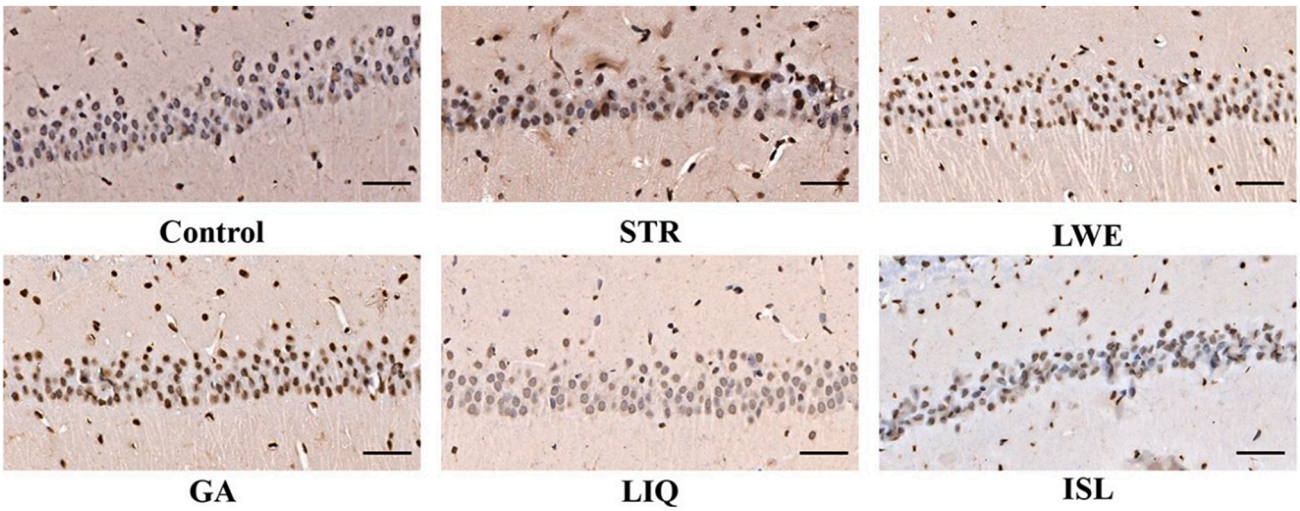
Heme oxygenase-1 (HO-1) is an inducible isoform of heme oxygenase (HO) enzymes, it has been reported to inhibit the release of HMGB1. [44] And it has been proved that SIRT1 could reduce the acetylation of HMGB1, which is a key step for HMGB1 nuclear export.[19] To further explore the reason why liquorice could inhibit HMGB1 release, the levels of HO-1 and SIRT1 in hippocampus were detected (Fig. 7A). As a result, HO-1 level in the STR group was significantly decreased compared with the control group ($p < 0.05$). In the LWE and the other three active components groups, HO-1 level was all increased in different degrees ($p < 0.05$). On the other hand, from the Western blot results, LWE could not reverse the decline of SIRT1 level caused by STR. GA and LIQ could restore the level of SIRT1 to the normal level ($p < 0.05$) while ISL could not. Then the acetylation status of HMGB1 was detected by coimmunoprecipitation (Fig. 7B). Results showed that the acetylation level of HMGB1 was greatly increased in the STR group ($p < 0.01$). LWE and the active components all reduced the acetylation degree of HMGB1 ($p < 0.05$). Among the monomers, GA exerted the greatest inhibiting effect ($p < 0.001$).

3.6. Liquorice affected the RAGE and TLR4 level

RAGE and TLR4 are the typical downstream receptors for HMGB1. As shown in Fig. 8A, there was an overexpression of RAGE and TLR4 in the STR group ($p < 0.05$). In the detoxification groups, the levels of RAGE were all downregulated ($p < 0.01$). In the aspect of TLR4 level, GA and ISL reversed the overexpression as compared with the STR group. Through co-immunoprecipitation, it was observed a strong interaction between HMGB1 with TLR4 in the STR group (Fig. 8B). Both LWE and the monomers showed the potential for inhibiting HMGB1 from binding with TLR4. However, we failed to detect the combination of HMGB1 and RAGE, probably because the combination of them was unstable and thus difficult to be shown in the IP results.

4. Discussion

Semen Strychni is classified as a highly toxic herb medicine according to Chinese pharmacopeia [18]. Intraperitoneal injection of *Semen Strychni* extract for 7 days induced obvious neurotoxicity and poor motor coordination in this study. About 10 min after administration with *Semen Strychni* extract at the dose of 0.2 g/kg, rats in the STR group began to appear poisoning symptoms like convulsion, opisthotonus and muscle rigidity as the literature reported [28]. We chose to record walking time as an indicator for evaluating motor coordination, which



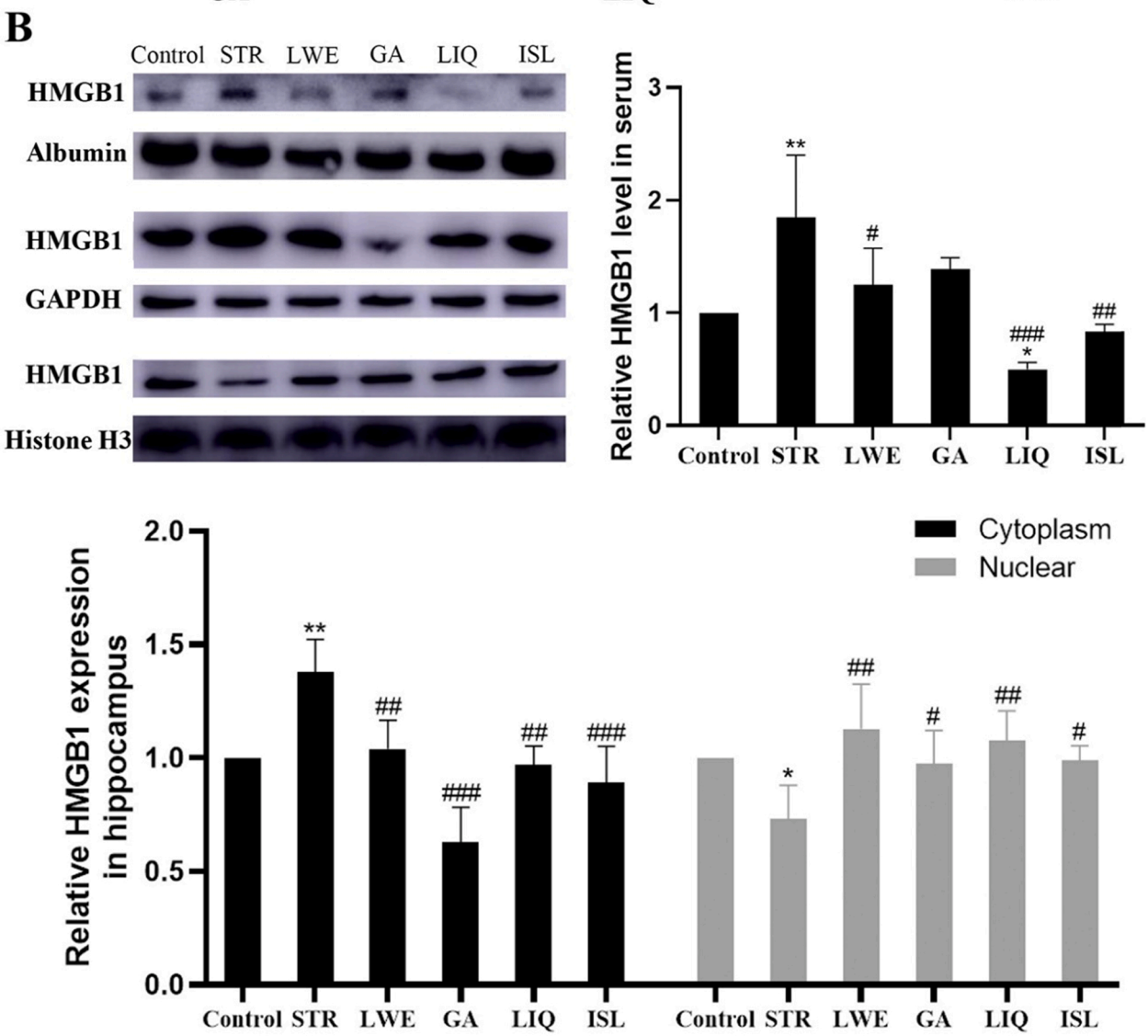


Fig. 6. The effects of S.Strychni and liquorice on HMGB1 distribution. (A) The immunohistochemical staining figure about expression and cellular distribution of HMGB1 in hippocampus in groups. (B) Representative immunoblot and densitometric analysis of HMGB1 expression in rat hippocampus in serum (top), hippocampus cytoplasm (middle) and hippocampus nuclear (bottom) from the control group, the STR group and four detoxification groups (LWE, GA, LIQ, ISL, n = 3). Data represent the ratio of HMGB1 and internal reference protein and are shown as mean ± SD. *p < 0.5, **p < 0.01 vs. the control group and #p < 0.5, ##p < 0.01, ###p < 0.001 vs. the STR group. Scale bar: 60 μm.

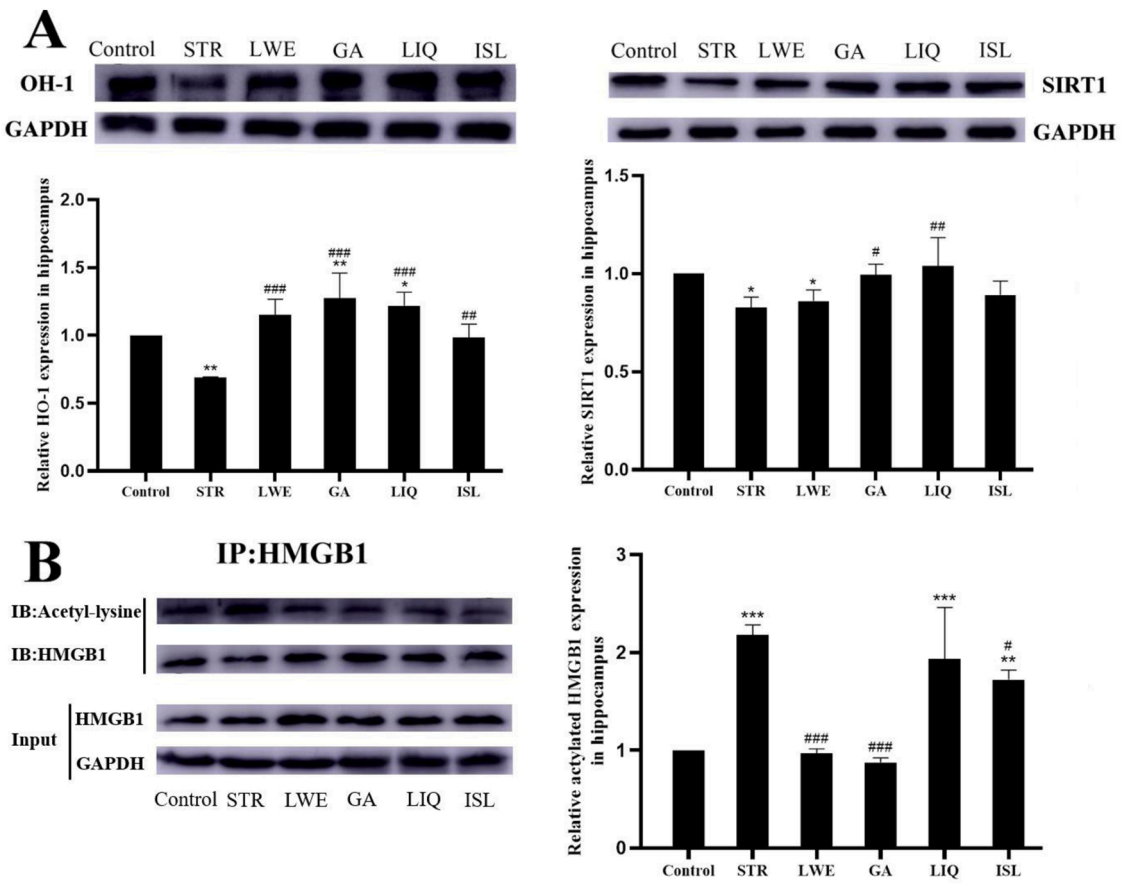


Fig. 7. (A) Two protein which may influence the circulating level of HMGB1 were assessed by Western blot. Representative immunoblot and densitometric analysis of OH-1 (right) and SIRT1 (left) expression in rat hippocampus from the control group, the STR group and four detoxification groups (LWE, GA, LIQ, ISL, n = 3). Data represent the ratio of the target protein and GAPDH and are shown as mean ± SD; (B) The acetylation of HMGB1 protein in the hippocampus tissues following Semen Strychni and liquorice treatment. The immuno-precipitates and total lysates were subjected to an immunoblot analysis. Representative Western blot of HMGB1 proteins, acetylation level and densitometric analyses. Data represent the ratio of acetylation level and HMGB1 and are shown as mean ± SD (n = 3). *p < 0.05, ***p < 0.001 vs. the control group and #p < 0.05, ##p < 0.01, ###p < 0.001 vs. the STR group.

could be more objective as compared to walking performance [42]. The results were consistent with the previous studies that high doses of *Semen Strychni* could induce motor disturbance by interfering with the balance of the neurotransmitters in brain [5,15]. An interesting phenomenon was that the walking time of rats in STR group became shorter as the experiment was conducted. On the seventh day of the experiment, there was no significant difference between STR group and three of the detoxification groups. This indicated that the damage of STR to motor coordination would improve with the increase of administration times. The reason for this may be some components in STR induced liver drug enzymes, so that the drug metabolism speed was accelerated and the toxicity was reduced in the later stage of administration. Immediate oral administration of LWE and its three active components post *Semen Strychni* injection relieved motor disturbance to a great extent. This may be a preliminary indication of the neuroprotective effect of liquorice against SAs toxicity.

Hippocampus is the target of various stressors and is seriously influenced by minute changes in brain homeostasis, hence we chose hippocampus to represent the whole brain status [48]. Similar to previous reports, our histopathological examination and TUNEL staining results showed that *Semen Strychni* caused organic damage and neuronal apoptosis in the hippocampus region [24]. There was obvious liquefactive necrosis and neuronal damage in the hippocampus in the STR group according to Fig. 2, which is consistent with our previous research that Semen Strychni induced neuronal degeneration in the hippocampal CA1 region.[45] It was reported that brucine and strychnine may downregulate Bcl-2 level and upregulate Bax level to exert cytotoxicity [57]. In this study, in addition to LIQ, which has been previously reported,[49] GA and ISL could also effectively reverse the overexpression of Bax in hippocampus. Interestingly, there was no significant reduction of Bax level in LWE group, although LWE was considered to include all three active monomers. The composition of LWE is very complex, we proposed that the components of liquorice can interact with each other to influence the absorption of the active ones. On the other hand, we evaluated the level of BDNF and NGF, the two most abundant neurotrophic factors in the CNS which can promote the survival of neurons [33]. At present, there is no recognized biomarker of Semen strychni. One study have examined nine potential neurotoxicity biomarkers in brain tissue of Semen Strychni-treated rats [35]. Their study found that the levels of BDNF and NGF in rat hippocampus decreased significantly after administration of Semen Strychni. Here, we used the levels of BDNF and NGF to characterize the neurotoxicity caused by Semen Strychni. Results showed that LWE had the weakest effect on BDNF and NGF induction. Besides, NSE was detected to estimate brain injury as a classical biomarker [53]. It was found that NSE concentration in serum was increased in the STR group, and the monomers all alleviated the brain damage induced by *Semen Strychni*. In addition, the oxidative stress and inflammation induced by *Semen Strychni* in the hippocampus were also relieved by the three active monomers, while LWE group showed no significant effect. To summarize, ISL was the most effective monomer in neuronutrition, while GA played an important role in preventing neuronal injury.

Researches on the specific molecular mechanism of *Semen Strychni*-induced neurotoxicity have so far proved inconclusive. Recently there

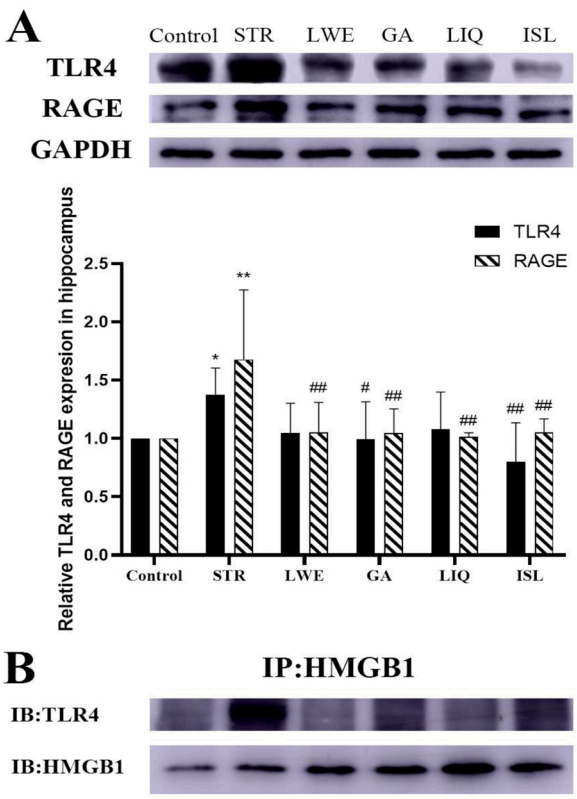


Fig. 8. (A) The expressions of two downstream receptors of HMGB1 – RAGE and TLR4 were assessed by Western blot. Representative immunoblot and densitometric analysis of RAGE and TLR4 expression in rat hippocampus from the control group, the STR group and four detoxification groups (LWE, GA, LIQ, ISL, n = 3). Data represent the ratio of the target protein and GAPDH and are shown as mean ± SD; (B) The physical interaction between TLR4 and HMGB1. The immunoprecipitates and total lysates were subjected to an immunoblot analysis Representative Western blot of HMGB1 and TLR4 proteins. *p < 0.05, **p < 0.01 vs. the control group and #p < 0.05, ##p < 0.01 vs. the STR group.

were plenty of reports indicated that HMGB1 could induce inflammation and apoptosis as a DAMP during many pathological processes, such as kidney injury, heart disease, liver fibrosis as well as neuroinflammation [8,20,31,43,47]. This reminded us that HMGB1 may act as a key factor in the process of neurotoxicity induced by *Semen Strychni*. In the present study, we measured the distribution of HMGB1 to explore it's function in *Semen Strychni* poisoning and the detoxification of liquorice. From the immunohistochemical staining result, there were more positive signals of HMGB1 around the neurons in the STR group. In the other groups, positive signals were more concentrated in the nucleus. Western blot was used to further determine the specific distribution. As a result, the HMGB1 level in the whole protein showed no significant difference among groups. Another article also reported the similar result [13], which indicated that HMGB1 level might not be a key point in the process of *Semen Strychni*-induced neurotoxicity. However, significant differences of HMGB1 location were observed in different groups. There was an incremental trend of HMGB1 in nucleus, cytoplasm and serum in the STR group. But in the LIQ and ISL groups, there were more HMGB1 concentrated in the nucleus. It was proved that GA could inhibit the translocation effectively but failed in inhibiting HMGB1 release into serum. Based on these results, we preliminarily confirmed that *Semen Strychni* induced HMGB1 releasing during neurotoxicity. However, for technical reasons, we failed to detect the content of HMGB1 in animal cerebrospinal fluid, so it's hard to tell the specific relationship between HMGB1 circulating levels and hippocampus levels in this project. There was a theory that HMGB1 released from brain cells can damage the BBB and increase its permeability, and can be released into the blood [36].

Many studies which focus on the HMGB1 efflux mechanism suggested that acetylation is the key step in the process of HMGB1 release ([12,54], [19]. During this process, SIRT1 mediates the deacetylation of HMGB1 to inhibit its release [16,30,38]. Studies showed that some of liquorice active components could induce or activate SIRT1 [11,52]. In our research, both GA and LIQ induced the increase of SIRT1 level and reduced the proportion of acetylated HMGB1 in consequence. Although the level of SIRT1 in LWE and ISL groups were not upregulated in the hippocampus, the proportion of acetylated HMGB1 was still downregulated. This might be explained that LWE and ISL mainly exert activation function. However, due to the deficiency of samples, we failed to detect the activation level of SIRT1. Furthermore, HO-1 is another factor that could influence the HMGB1 release [44]. It was illustrated that HO-1 level could affect the circulating level of HMGB1 [21]. Similarly, the HO-1 level was also upregulated by LWE and the three active monomers, which could be one factor to determine the circulating levels of HMGB1, and this result was obviously correlated with the HMGB1 distribution result. Taken together, we found that GA exerted the greatest effect on HMGB1 nucleus export, while LIQ and ISL developed a larger impact on the exocytosis of HMGB1.

HMGB1 functions as a DAMP mainly by interacting with its receptors like RAGE and TLR4 [22]. In this experiment, *Semen Strychni* significantly upregulated the level of RAGE and TLR4. This is the first time that we discover the inducing function of *Semen Strychni* on the two receptors. It is discovered that the RAGE promoter contains multiple functional NF-κB binding site, proinflammatory cytokines can promote the expression of RAGE, potentially triggering a receptor-dependent autoinflammatory loop [17,37]. In this study, Semen Strychni caused significant neuroinlammation and activated the HMGB1-RAGE axis. The proinflammatory factors produced after STR treatment may further increased the expression of RAGE. On this basis, STR may induce pro-inflammatory polarization of microglia through the activation of HMGB1-RAGE-NF-κB pathway [6]. In addition, other drugs like lipopolysaccharide (LPS) which is well known for the capability to generate neuroinflammation can induce RAGE and TLR4 levels [20,51]. Therefore, we speculated that *Semen Strychni* may affect the HMGB1-RAGE/TLR4 axis to induce neurotoxicity just like LPS. It has been demonstrated that GA could bind to HMGB1 directly and inhibit extracellular HMGB1 cytokine activities to play a neuroprotective role [25,40,43]. On closer analysis, we found that the binding site of GA with HMGB1 overlapped the binding site of TLR4 [12]. According to the result of co-immunoprecipitation, there was a large amount of HMGB1-TLR4 conjugates in the STR group. In the detoxification groups, the connection of HMGB1 and TLR4 was relatively weak. Interestingly, except for GA, LIQ and ISL can also inhibit this connection even there was no proof of the interactions between HMGB1 and LIQ or ISL. However, researchers suggested that reduced HMGB1 and oxidized HMGB1 may interact with different receptors and have altered DNA binding activities [9]. Due to the anti-oxidation property of liquorice, the cellular redox status may influence the structure, location, and function of HMGB1. In terms of RAGE, we failed to detect the conjugate of HMGB1 and RAGE in the co-immunoprecipitation result, despite some other reports showed that HMGB1 had indeed a connection with RAGE [27]. Therefore, more experiments and discussions are needed in the future to explain why this interaction was not detected.

The detoxification mechanism of glycyrrhizin extract and its related active monomers (glycyrrhizic acid, liquiritigenin and isoglycyrrhizin) was explored through the establishment of an acute neurotoxic rat model of *Semen Strychni* in this project. The poisoned rats showed decreased motor coordination ability, aggravated oxidative stress, inflammatory reaction and neuronal apoptosis in the brain hippocampus, which was alleviated by the application of liquorice and its active monomers. This study preliminarily proved that HMGB1 was released in the neurotoxic pathological process of SAs. Liquorice and its active monomers mainly inhibited the release of HMGB1 and its pro-inflammatory effect through inhibition of SIRT1 and up-regulation of

HO-1. On the other hand, by down-regulating the level of the downstream receptors RAGE and TLR4 and their binding with HMGB1, the proinflammatory signal was cut off from further downstream transmission and the cascade amplification was effectively prevented.

5. Conclusion

In this study, we established a rat model of neurotoxicity induced by Semen Strychni. Then the detoxification of LWE and its three active components including GA, LIQ and ISL were verified. Different from our expectations, LWE which contains all the three active monomers did not exert the best detoxification effect. This might be the result of interactions between its various components. Hence, we hypothesize that monomer administration might be more controllable and more targeted. Besides, each active monomer plays a detoxification role from different aspects, as the combined application of several active monomers may increase the detoxification from different angles. Through this research, we first demonstrated that HMGB1 participated in the process of Semen Strychni-induced neurotoxicity and the active components of liquorice could inhibit the translocate and binding process of HMGB1 in the detoxification process. This study provided a new direction and target on solving Semen Strychni toxicity. At the meantime, it also made a deeper exploration on the underlying mechanism of liquorice detoxification.

CRediT authorship contribution statement

Duan Xiaoyu: Conceptualization, Methodology, Data curation, Investigation, Formal analysis, Writing – original draft preparation. **Wen Jing**: Methodology, Investigation, Writing – original draft preparation, Data curation, Software. **Fang Pingfei**: Methodology, Supervision. Writing – reviewing and editing. **Zhang Min:** Visualization, Writing – reviewing and editing. **Wang Chao:** Investigation, Writing – reviewing and editing. **Xiang Yalan:** Investigation, Writing – reviewing and editing. **Wang Lu** : Writing – reviewing and editing. **Yu Changwei**: Writing – reviewing and editing. **Yan Miao**: Writing – reviewing and editing. **Zhang Bikui**: Writing – reviewing and editing.

Data availability

The data that has been used is confidential.

Acknowledgments

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Conflicts of interest

There are no conflicts to declare.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.112884.

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