Glycyrrhizic Acid alleviate the Neurotoxicity of Semen Strychni through inhibition of HMGB1 phosphorylation and inflammatory

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Research Article

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Abstract

Backgrounds: In recent years, the neurotoxicity of Semen Strychni was reported in many clinical cases. Hence, this study was conducted to investigate the role of HMGB1 in the model of neurotoxicity induced by Semen Strychni and the alleviation of glycyrrhizic acid, which is related to the regulation of HMGB1 release.

Methods: The rats were intraperitoneally injected with Semen Strychni extract (175mg/kg), followed by oral administration of glycyrrhizic acid (50 mg/kg) for 4 days. After treatment, neuronal degeneration, apoptosis, and necrosis were observed by Nissl, FJB, and Tunel. ELISA was used to detect related inflammatory indicators TNF-α, IL-1β, HMGB1, and neurotransmitter enzymes MAO, AChE. IF and Western Blot were used to determine the localization of HMGB1, p-HMGB1, and NF-κB in the nucleus and cytoplasm; CO-IP was used to determine the interaction between PP2A, PKC, CaMKIV and HMGB1. The expression of ERK, p-ERK, JNK, p-JNK, p38, p-p38, was detected by Western Blot.

Results: After neurotoxic model rats were treated with glycyrrhizic acid neuronal damage and apoptosis were improved to a certain extent, the inhibitory effect of Semen Strychni on neurotransmitter metabolic enzymes was removed, and the levels of inflammatory indexes TNF-α and IL-1β were also significantly down-regulated. The results of IF and Western Blot showed that Semen Strychni reduced the nucleus level of HMGB1, significantly increased the cytoplasm and serum release levels, and increased the level of p-HMGB1, while glycyrrhizic acid could down-regulate the level of p-HMGB1, inhibit the nucleoplasmic shuttle and extracellular release process of HMGB1. Glycyrrhizic acid could strengthen the interaction between HMGB1 and PP2A and reduce the combination of HMGB1 and PKC. Glycyrrhizic acid significantly down-regulated the expression levels of p-ERK, p-JNK, and p-p38 proteins, and inhibited the transfer of NF-κB from the cytoplasm to the nucleus.

Conclusions: Glycyrrhizic acid has a certain ameliorating effect on the neurotoxicity induced by Semen Strychni, and its mechanism is related to the inhibition of HMGB1 phosphorylation, the reduction of nucleoplasmic shuttle, and the inhibition of downstream inflammatory pathways of HMGB1.

Introduction

Semen Strychni is obtained from the dried mature seeds of the medical plant Strychnos Nux Vomica, which have been found to have a variety of pharmacological activities such as anti-tumor, anti-inflammatory, immunosuppression, and nerve excitation[1-4]. However, overdose or long-term use could lead to multiple organ damage, especially neurotoxicity, which limits its clinical application[5, 6]. Studies have shown that brucine and Semen Strychni are the main bioactive and toxic substances[7]. The toxic dose of Semen Strychni can cause the over-limit inhibition of the cerebral cortex, the excitation of spinal cord reflex function, and the forced contraction of respiratory muscle, and finally lead to cardiac arrest or asphyxia[6]. As for brucine, its toxic dose can block neuromuscular transmission and cause curare like symptoms[6]. According to recent researches, Semen Strychni poisoned rats have severe oxidative stress...
and inflammation in the brains\cite{8-10}. Therefore, it is crucial to elucidate the neurotoxicity mechanism of strychnine and find effective antidote.

In recent years, much work has been carried out on the mechanism of licorice alleviating the toxicity of Semen Strychni. It is reported that the combination of licorice and Semen Strychni can significantly reduce the contents of Semen Strychni and brucine in Semen Strychni. Chen et al.\cite{11} revealed the renal protective mechanism of licorice on Semen Strychni at the pharmacokinetic level, that is, reducing AUC and C_{max} of Semen Strychni and brucine. The research of cell metabolomics and animal metabolomics explains the hepatorenal toxicity mechanism of Semen Strychni from the aspects of energy metabolism, ion transport system disorder, abnormal glycolysis and amino acid metabolism\cite{12, 13}. However, there are few studies on the mechanism of neurotoxicity. Therefore, the neurotoxic mechanism of Semen Strychni is worthy of further exploration, and the detoxication effect of licorice should be further clarified.

Glycyrrhizic acid, as a natural anti-inflammatory and antiviral triterpenoid, mainly for the treatment of liver diseases, and also as a cellular immune modulator\cite{14}. Glycyrrhizic acid was found to have neuroprotective effects in glutamate-induced cytotoxicity, which may serve as a potential therapeutic agent for neurodegenerative diseases\cite{15, 16}. Glycyrrhizic acid can also inhibit the activation of microglia and induction of pro-inflammatory cytokines in post-ischemic middle cerebral artery occlusion, and improve movement disorders and neurological deficits\cite{17}, which suggest that glycyrrhizic acid has good prospects in neuroprotection. In addition, we found that the pharmacological effects of glycyrrhizic acid in neurological diseases or brain injury are closely related to the high-mobility group box 1 (HMGB1).

HMGB1 accumulated in the serum of epilepsy model mice and down-regulated after administration of glycyrrhizic acid\cite{18}; the improvement of glycyrrhizic acid on stroke\cite{19} and intracerebral hemorrhage\cite{20} was also related to HMGB1 protein; more scholars proposed HMGB1 as one of the sources of early brain injury and cerebral vasospasm after subarachnoid hemorrhage. Whether the mechanism of Semen Strychni neurotoxicity and the neuroprotective of glycyrrhizic acid were mediated by HMGB1 is unknown. There are two mechanisms for the release of HMGB1: one is actively secreted by monocytes/macrophages and mature dendritic cells under stimuli such as injury, inflammation and infection; the other is passively released by necrotic cells and damaged cells. Extracellular HMGB1 acts as an endogenous danger signal molecule and is involved in the pathogenesis of various diseases, including sepsis\cite{21}, acute and chronic liver injury\cite{22}, kidney disease\cite{23}, cerebral ischemia\cite{24} and neuropathy\cite{25}. The main mechanism is to activate the downstream NF-κB and MAPKs pathways by binding to Toll-like receptors and RAGE, further inducing the secretion of inflammatory cytokines. High levels of inflammatory factors in turn stimulate non-parenchymal cells to secrete HMGB1. Therefore, HMGB1 can not only directly participate in tissue damage as a pro-inflammatory factor, but also act as an inflammatory mediator to aggravate the inflammatory cascade\cite{26}. Therefore, we speculate that reducing the release of HMGB1 will be a key therapeutic target. However, the mechanism how HMGB1 is translocated from the nucleus to the cytoplasm and released outside the cell is not fully cleared. Highly phosphorylated HMGB1 will be easier to transfer from the nucleus to the cytoplasm, so the
phosphorylation of HMGB1 is considered to be the key to its extracellular release\textsuperscript{[27]}. Studies have reported that calmodulin dependent protein kinase IV (CaMKIV)\textsuperscript{[28]} and protein kinase C (PKC)\textsuperscript{[29]} can phosphorylate HMGB1, while protein phosphatase 2A (PP2A) can dephosphorylate the phosphorylated HMGB1, thereby reducing the nucleocytoplasmic shuttle and subsequent extracellular release of HMGB1\textsuperscript{[30]}. Glycyrrhizic acid has been proved to be a natural inhibitor of HMGB1, which can inhibit the activity of extracellular cytokines of HMGB1. Therefore, we further explore whether glycyrrhizin inhibits HMGB1 nucleocytoplasmic shuttle by regulating HMGB1 phosphorylation, so as to play a neuroprotective role.

In this paper, we explored the neurotoxicity of Semen Strychni mediated by HMGB1, and then looked for possible ways to alleviate the toxicity by adding HMGB1 inhibitor glycyrrhizic acid. This study confirmed that HMGB1 is involved in the neurotoxicity of Semen Strychni and the anti neurotoxicity of glycyrrhizic acid. This study aims to provide reference for improving the clinical safety of Semen Strychni.

Materials And Methods

2.1 Plant material

Semen Strychni (batch number 201022891) were obtained from SanXiang Co. Ltd. (Changsha, China). All the herbs were authenticated by Professor Yu-hua Wang (Department of Pharmacy, the Second Xiangya Hospital, Central South University). Voucher specimens of the two herbs were deposited at Department of Pharmacy, the Second Xiangya Hospital.

2.2 Extraction of herbs

After the raw Semen Strychni seeds were crushed, a proper amount of powder was taken and soaked in 75% acidic ethanol (pH=5, 1:12, W/V) for 12 h, and heat to reflux for three times, each for 1 h. The extraction was filtered using a 0.45 μm microporous filter membrane under hot conditions. Then the filtrate was combined and concentrated by a rotary evaporator until all ethanol was volatilized. The filtrate pH was adjusted to 6.5 with 0.5 mol/L NaOH. The extract was mixed with 1% carboxymethylcellulose sodium (CMC-Na) solution and water to prepare 50 mg raw Semen Strychni/0.5%CMC-Na ml. 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)

2.3 Animals Experiments

The experimental animals were SPF-grade male SD rats, purchased from Hunan SJA Laboratory Animal Co., Ltd., with the license number SCXY (Xiang) 2019-0004. The rats were raised in the Department of Laboratory Animal Science of Central South University, and the experimental unit’s animal use license number is SYXK (Xiang) 2015-0017. This study was reviewed and approved by the Laboratory Animal Management Committee of the Department of Laboratory Animal Science of Central South University
Thirty-eight rats were used to assess the renal injury induced by Semen Strychni and randomly divided into 4 groups (n=12) as follows:

**Blank group (Control):** Rats were intraperitoneally injected with 0.5% CMC Na solution with a volume of 3.5 ml/kg. After the injection, 0.5% CMC-Na solution was given by intragastric administration at a volume of 5 ml/kg.

**Strychni poisoning group (SS):** Rats were intraperitoneally injected with strychni extract (0.05 g/ml) with a volume of 3.5 ml/kg. Immediately after the injection, 0.5% CMC-Na solution was given by intragastric administration with a volume of 5 ml/kg.

**Low-dose glycyrrhizic acid detoxication group (SS+LGA):** Rats were intraperitoneally injected with strychni extract (0.05 g/ml) in a volume of 3.5 ml/kg, and gavage a low-concentration glycyrrhizic acid solution immediately after the injection (15 g/ml). mg/ml), the administration volume is 5 ml/kg.

**High-dose glycyrrhizic acid detoxication group (SS+HGA):** Rats were intraperitoneally injected with strychni extract (0.05 g/ml) with a volume of 3.5 ml/kg. Gavage with low-concentration glycyrrhizic acid solution (30 mg/ml), the administration volume is 5 ml/kg.

**2.4 Samples collection**

At the time of dissection, the blood was collected from the heart, centrifuged (4°C, 3500 rpm) for 10 min. The supernatant was extracted to obtain serum. The wholly separated brain tissues of rats were randomly selected, soaked, and cleaned with normal saline several times and then put into a centrifuge tube with paraformaldehyde.

**2.5 Histopathological examination**

**2.5.1 Nissl staining**

The rat brains were fixed with 4% paraformaldehyde and embedded in paraffin. The paraffin was then sectioned, dewaxed, and stained with toluidine blue after hydration. The sections were placed in xylene solution for 5 min to make them transparent, and then mounted with neutral gum. Images of the rat hippocampus were collected under a microscope and analyzed.

**2.5.2 TUNEL staining**

After paraffin embedding, sectioning, dewaxing and other steps in the same way as above, proteinase K working solution was added dropwise for antigen retrieval, followed by membrane rupture, labeling with TdT :d UTP at 1:9, DAPI counterstaining, and anti-fluorescence quenching. After mounting with anti-mounting medium, the tissue sections were observed under a fluorescence microscope and images were collected.

**2.5.3 FJB staining**
After dewaxing and hydrating the paraffin sections, FJB dilution was prepared according to the ratio of 50% glacial acetic acid to 1:400 for staining. The nuclei were stained with DAPI and then placed in xylene solution to seal the sections with transparent neutral resin. The tissue sections were then observed under a fluorescence microscope and images were collected.

2.6 ELISA

The collected blood was centrifuged at 2500 rpm for 15 minutes at 4°C, and serum was collected for ELISA detection. AChE, MAO, HMGB1, TNF-α, IL-1β were quantitatively detected according to the instructions.

2.7 Western blot

Total and cytoplasmic proteins were isolated from brain tissue samples. Proteins were run on 10% or 12% polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. PVDF membranes were blocked with 5% BSA for 60–90 min at RT and incubated with antigen-specific primary antibodies overnight at 4°C. Blots were then incubated with species-specific secondary antibodies for 60 min at RT. Proteins were visualized by incubation with a chemiluminescent substrate kit. The primary antibodies used in this study were anti-HMGB1 (10829-1-AP, Proteintech, USA), anti-p-Serine (ab9332, Abcam, UK), anti-NF-κB (10745-1-AP, Proteintech, USA), anti-p-38 (14064-1-AP, Proteintech, USA), anti-PP2A (13482-1-AP, Proteintech, USA), anti-PKC (21991-1-AP, Proteintech, USA), anti-CaMKIV (13263-1-AP, Proteintech, USA).

2.8 Immunofluorescent staining.

The paraffin sections were dewaxed and hydrated, and autofluorescence quenchers were added dropwise to the tissue sections after antigen retrieval. BSA solution was added dropwise for blocking. The primary antibody was added overnight at 4 °C; the secondary antibody was added and reacted at room temperature for 50 min. DAPI staining solution was added dropwise on the tissue to counterstain the nuclei. Mount slides with anti-fluorescence quenching mounting medium. The tissue sections were then observed under a fluorescence microscope and images were collected.

2.9 CO-IP

Total protein was extracted from each group and stored at −80°C. HMGB1 antibody was added to the protein supernatant and the antigen-antibody mixture was shaked slowly at 4°C, and incubated overnight. Take Protein A/G agarose beads, add IP lysis buffer, and centrifuge at 3000 rpm to retain the pellet. The cell lysate incubated with the antibody overnight was added to the pretreated Protein A/G agarose beads to couple the antibody to the Protein A/G agarose. After co-immunoprecipitation, centrifuge at 3000 rpm for 3 min at 4 °C, aspirate the supernatant, and collect the pellet. Expression of p-HMGB1 with PP2A, PKC and CaMKIV was assessed using Western blot analysis.

2.10 Statistical Analysis
SPSS 18.0 software was used for statistical analysis of data. Data were presented as mean ± standard error (mean ± SEM). One-way analysis of variance (ANOVA) was used for comparison between multiple groups, and then LSD test was used for comparison between two groups. When P<0.05, the difference was considered to be statistically significant. The graphing software used was GraphPad Prism 8.0.

Results

3.1 Glycyrrhizic acid relieves neuronal damage caused by Semen Strychni

Figures 4-4 are the results of Nissl staining of neurons in the CA1 region of the hippocampus of rats in each group. As can be seen from the figure, compared with the Control group, the neurons in the SS group were greatly reduced, the cell integrity was lost, the distribution of Nissl bodies was uneven, and the staining was blurred (3-1-B), indicating that the strychnids caused the hippocampal neurons damage. After treatment with glycyrrhizic acid, the neuronal damage in the SS + LGA group (Fig. 3-1-C) and SS + HGA group (Fig. 3-1-D) was relieved, it showed that the number of Nissl bodies increased and the staining was darker, and the neurons were arranged more closely. FJ-B histofluorescence staining is a sensitive technique to detect neuronal degeneration. As shown in Figure 3-2-A and Figure 3-2-E, almost no degeneration and necrosis were found in the hippocampal CA1 and CA3 regions of the rats in the Control group. Compared with the control group, the number of FJB-positive cells in the Semen Strychni neurotoxicity model group increased, especially in the CA3 area (Fig. 3-2-F). Very few were seen in the CA1 and CA3 regions of the SS + LGA group (Fig. 3-2-C, Fig. 3-2-G) and the SS + HGA group (Fig. 3-2-D, Fig. 3-2-H). There was no significant difference in FJB positive neurons compared with the Control group, indicating that glycyrrhizic acid has an improving effect on nerve cell necrosis. The results of TUNEL staining are shown in Figure 3-3. It can be seen from the figure that there are a small number of TUNEL-positive cells in the cortex of the rats in the Control group, while the number of positive cells in the SS group is significantly increased, and a large number of apoptotic cells are distributed. The overall distribution of apoptotic cells in the SS + LGA group and the SS + HGA group was less than that in the SS group, suggesting that glycyrrhizic acid has a certain protective effect on neuronal apoptosis induced by strychni.

3.2 Glycyrrhizic acid relieves disturbance of neurometabolic enzyme levels caused by Semen Strychni

Figures 3-4 show the levels of AChE and MAO in the serum of rats in each group. After administration of strychnids, the levels of AChE and MAO in the serum of the rats in the SS group were significantly lower than those in the control group (p<0.01). High doses of glycyrrhizic acid significantly up-regulated AChE levels (p < 0.05). As for MAO, both high and low doses of glycyrrhizic acid significantly increased the level of MAO (p < 0.05), which fully demonstrated that glycyrrhizic acid can effectively regulate the
neurotransmitter metabolism disorder caused by strychnids, and make the levels of related enzymes return to normal.

3.3 Glycyrrhizic acid inhibits HMGB1 release and nucleocytoplasmic translocation

Figures 3-5 show the content of HMGB1 in serum, that is, the level of extracellular release of HMGB1. Compared with the control group, the extracellular HMGB1 level in the SS group was significantly increased (p < 0.05), which indicated that strychnids could increase the release of HMGB1. After administration of glycyrrhizin acid, HMGB1 recovered to the level of the blank group (p<0.05). Figure 3-6-A shows that the expression of HMGB1 is basically distributed in the nucleus, and there is almost no positive signal outside the nucleus. In the SS group (Fig. 3-6-B), there was obvious nucleocytoplasmic shuttling phenomenon. HMGB1 was not limited to the nucleus, and the distribution of HMGB1 in the cytoplasm was greatly increased. Compared with the SS group, the cytoplasmic distribution of HMGB1 in the SS+LGA group was improved to a certain extent, while in the SS+HGA group, the inhibitory effect of glycyrrhizin acid on Semen Strychni-induced nucleocytoplasmic transfer was more obvious, and HMGB1 was mainly distributed in the nucleus. Subsequently, the protein expression levels in the nucleus and cytoplasm were further verified by Western Blot technology. The results reflected in Figures 3-7 are generally consistent with immunofluorescence. For HMGB1 in the nucleus, its expression was the lowest in the SS group, while high-dose glycyrrhizin acid significantly increased the expression level of HMGB1 in the nucleus (p < 0.01). The expression of HMGB1 in the cytoplasm was just the opposite. The cytoplasmic level of HMGB1 in the injury group was higher than that in the blank group. High-dose glycyrrhizin acid could reverse this situation and reduce the cytoplasmic HMGB1 level to below the normal level (p<0.01).

3.4 Glycyrrhizic acid regulates the interaction of dephosphorylase and phosphokinase with HMGB1 to inhibit phosphorylation of HMGB1 induced by Semen Strychni

Figures 3-8-A and 3-8-B are the phosphorylation levels of HMGB1 in the nucleus and cytoplasm, respectively. Compared with the blank group, the phosphorylation levels in the nucleus and cytoplasm of the strychnids group were significantly increased (p < 0.01). Glycyrrhizic acid can significantly inhibit the phosphorylation of HMGB1. Figure 3-9-A uses HMGB1 as the bait protein to precipitation to detect the expression of the protein PP2Ac, while Figure 3-9-B verifies the binding between PP2Ac and HMGB1 in reverse. The results showed that the interaction between PP2Ac and HMGB1 was weakest in the Semen Strychni subgroup. Compared with the Semen Strychni group, the binding of PP2Ac to HMGB1 was increased in the glycyrrhizin acid group. Figures 3-9-C and 3-9-D are the results of detecting the binding of
The binding of HMGB1 to PKCα. The binding of HMGB1 to PKCα was enhanced after administration of Semen Strychni, but this binding was partially blocked by glycyrrhizic acid.

### 3.5 Glycyrrhizic acid inhibits the release of inflammatory factors and the activation of inflammatory pathways induced by Semen Strychni

Figures 3-10 show the levels of pro-inflammatory factors TNF-α and IL-1β in the serum of rats in each group. In the Control group, the concentrations of TNF-α and IL-1β were both at lower levels. After administration of Semen Strychni, the concentrations of TNF-α and IL-1β in the serum of rats in the SS group were significantly increased (p < 0.05), indicating that Semen Strychni can promote the release of inflammatory factors and aggravate the inflammatory response. After glycyrrhizic acid intervention, the TNF-α concentration in the SS+LGA group and the SS+HGA group was significantly lower than that in the SS group (p<0.01), indicating that glycyrrhizic acid can reverse the release of pro-inflammatory factors caused by Semen Strychni, and has a certain effect. Anti-inflammatory effect. In addition, high-dose glycyrrhizic acid significantly down-regulated IL-1β levels (p < 0.05).

The MAPKs pathway mainly includes JNK, p38 and ERK pathways, and the phosphorylation levels of the three proteins represent their activation. As shown in Figure 3-11-A and Figure 3-11-B, the phosphorylation levels of JNK and p-38 in the SS group were significantly higher than those in the Control group (p<0.05). Compared with the SS group, the SS+LGA group could reverse this change and down-regulate the phosphorylation levels of JNK and p-38, especially the high-dose glycyrrhizic acid group could down-regulate the phosphorylation levels of the two to below the blank group. The immunofluorescence results of NF-κB (Fig. 3-11) showed that many NF-κB positive signals were seen outside the nucleus of the cells of the Control group, and there was less distribution in the nucleus. In contrast, almost all NF-κB in the SS group was distributed in the nucleus. After glycyrrhizic acid administration, the extranuclear distribution of NF-κB was increased in SS+LGA and SS+HGA groups.

### Discussion

Glycyrrhizic acid has a certain protective effect on the neurotoxicity caused by Semen Strychni. Previous studies have shown that Semen Strychni poisoning causes a large number of apoptosis, loss and degeneration of neuronal cells, cell morphological variation, disordered cell arrangement, and liquefied necrosis in local tissues\(^9,12\). Consistent with the report, the histopathological section of this experiment showed that the symptoms of neuronal degeneration, necrosis and apoptosis occurred in the hippocampus and cortex of rats after continuous administration of Semen Strychni. After administration of glycyrrhizic acid, the above symptoms were improved to a certain extent, suggesting the neuroprotective effect of glycyrrhizic acid. However, from the slice results alone, the detoxification effect of high-dose glycyrrhizic acid does not seem to be significantly better than that of low-dose glycyrrhizic acid. This experiment also measured the enzymes related to neurotransmitter metabolism, Mao and
AChE. It was found that Semen Strychni can significantly down regulate the levels of Mao and ache, suggesting that the metabolism of monoamine neurotransmitters and acetylcholine is inhibited, resulting in the accumulation of acetylcholine and serotonin. Excessive acetylcholine can activate neuronal nicotinic acetylcholine receptors, stimulate voltage-gated calcium channels, cause the accumulation of cytoplasmic calcium, and then trigger epilepsy [31, 32]. It is speculated that the epilepsy like symptoms of Semen Strychni are caused by the accumulation of acetylcholine. After detoxification with glycyrrhizic acid, the down-regulated MAO and ACHE were reversed, indicating that glycyrrhizic acid can alleviate neurotoxicity by regulating abnormal neurotransmitter metabolism. At the same time, by investigating the neuroinflammatory reaction caused by Semen Strychni poisoning and the anti-inflammatory protective effect of glycyrrhizic acid, it was found that glycyrrhizic acid could antagonize the pro-inflammatory factor TNF caused by Semen Strychni-α and IL-1β. It can reduce the inflammatory level of the body.

HMGB1 nucleocytoplasmic shuttle and extracellular release are regulated by HMGB1 phosphorylation. HMGB1 located in the nucleus, as a chromatin binding factor, is involved in DNA bending and gene expression regulation. Extracellular HMGB1 can be used as a damage related signal molecule to participate in the pathogenesis of a variety of diseases. In sepsis rats [33], chronic obstructive pulmonary disease (COPD) [34] and nonalcoholic fatty liver disease (NAFLD) [35], intranuclear HMGB1 was induced to transfer to the cytoplasm and accumulate outside the cell. It can be seen that the localization of HMGB1 plays a decisive role in its physiological function. Therefore, this experiment first measured the serum level of HMGB1 in rats in each group. The results showed that compared with the blank group, the release of HMGB1 in the poisoning group increased, indicating that HMGB1 was indeed involved in the process of nerve injury caused by Semen Strychni. Secondly, if diagram and Western blot results further showed that the injury could promote the transfer of HMGB1 in the nucleus to the cytoplasm, reduce the nuclear retention level and increase the cytoplasm. Glycyrrhizic acid, which can resist injury, can inhibit the nucleocytoplasmic transfer of HMGB1 and increase the nuclear retention level, which verifies the inflammatory and damaging effects of extracellular HMGB1. It is reported that [27, 30], the phosphorylation state of serine in the NLSS sequence of HMGB1 is the first step of its extracellular secretion. When serine residues are dephosphorylated or unmodified, HMGB1 binds tightly to chromatin and is not released into the cytoplasm. On the contrary, highly phosphorylated HMGB1 reduces its binding activity to DNA by 10 times due to more negatively charged groups, resulting in the transfer of HMGB1 from the nucleus to the cytoplasm and release to the outside of the cell [36]. Consistent with the above view, this experiment found that with the increase of HMGB1 cytoplasmic transfer and release, its phosphorylation level in nucleus and cytoplasm also increased accordingly. If the phosphorylation level decreases, HMGB1 release decreases. Therefore, we believe that the cytoplasmic transport of HMGB1 is the result of its phosphorylation, but the specific regulatory mechanism of phosphorylation has not been clarified. Taira et al. [30] found in RAW264 there is an interaction between HMGB1 and PP2A in 7 cells, and PP2A can dephosphorylate specific phosphoserine residues in NLSS. Replacing serine residues with alanine weakened the inhibitory effect of PP2A on HMGB1 cytoplasmic transfer, suggesting that PP2A may be related to HMGB1 phosphorylation and nucleocytoplasmic shuttle. At the same time, some scholars have carried out a lot of screening work on phosphokinase. PKC and CaMKIV kinases have been confirmed to
mediate HMGB1 serine phosphorylation. It is speculated that the phosphorylation of serine residues in HMGB1 sequence may be jointly regulated by protein kinase and dephosphorylase, which is not determined by a single factor, which has also been verified in this experiment. We found that HMGB1 interacted with PP2A and PKC under the administration of Semen Strychni and glycyrrhizic acid, and glycyrrhizic acid enhanced the interaction between HMGB1 and PP2A, but weakened the binding between HMGB1 and PKC. This may be due to the conformational change of HMGB1 induced by the direct binding of glycyrrhizic acid to HMGB1\textsuperscript{[37]}, which hinders the interaction with PKC and promotes the binding with PP2A. Secondly, the binding site of glycyrrhizic acid in HMGB1 is very close to the phosphorylation site of PKC in HMGB1\textsuperscript{[38]}, which may block the phosphorylation of serine residues by PKC. On the other hand, the study said that\textsuperscript{[39]} PP2A can promote the growth and development of neurons by dephosphorylating some proteins involved in neuronal growth, which just explains the neuroprotective effect of glycyrrhizic acid. Unfortunately, no binding compound between HMGB1 and CaMKIV was found in this experiment. It is speculated that the binding of the two proteins is weak or the binding compound is unstable and has not been detected. In a word, through layers of experimental verification, we can preliminarily draw the following conclusions: the occurrence of Semen Strychni neurotoxicity indeed involves HMGB1, and glycyrrhizic acid can inhibit its phosphorylation level and reduce nucleocytoplasmic shuttle and extracellular release by regulating the interaction between HMGB1, PP2A and CaMKIV.

RAGE receptors and TLRs receptors (mainly TLR-4 and TLR-2) are two key receptors for extracellular HMGB1 to play the role of inflammatory factors. By binding to receptors, HMGB1 activates NF-κ B. Promote NF-κ B translocates from cytoplasm to nucleus and induces cascade transcription of proinflammatory cytokines and chemokines. MAPKs signaling pathway mainly includes extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK. This series of parallel cascade serine/threonine kinases are also activated by extracellular HMGB1, leading to inflammatory response. In addition, high levels of inflammatory molecules can feed back and promote the additional release of HMGB1 by immune cells, aggravating the injury\textsuperscript{[40,41]}.

As a natural inhibitor of HMGB1, glycyrrhizic acid can inhibit its extracellular cytokine activity. There is evidence\textsuperscript{[42]} that glycyrrhizic acid can inhibit ERK, p38 and NF-κ B Activation of B improves renal injury and inflammatory response induced by diabetes. Glycyrrhizic acid has also been shown to play a protective role in cardiac ischemia-reperfusion injury by blocking HMGB1 dependent p-JNK / Bax pathway\textsuperscript{[39,43]}. The results showed that glycyrrhizic acid reversed the increase of JNK and p38 phosphorylation levels caused by Semen Strychni and inhibited the transfer of NF-κ B to the nucleus, which could reduce the amplification process of inflammatory cascade and partially alleviate the neurotoxicity of Semen Strychni. we believe that blocking the activity of HMGB1 extracellular cytokines is an effective therapeutic strategy.

In addition to the direct regulation of pathway, the study found that this pathway is also regulated by PP2A and PKC, which play negative and positive regulatory roles respectively. These kinases play an
indispensable role in the growth and development of neurons\textsuperscript{[39]}. Although there is no direct evidence that HMGB1 plays an intermediate link role in the regulation of PP2A and PKC on downstream pathways, it is certain that glycyrrhizic acid inhibits MAPKs and NF-κB pathway activation in either pathway.

In conclusion, this study confirmed that glycyrrhizic acid has a certain improvement effect on the inflammatory response, neuronal apoptosis and degeneration and neurotransmitter metabolism disorder caused by Semen Strychni. At the same time, it is clear that HMGB1 plays a key role in the process of nerve injury caused by Semen Strychni, and glycyrrhizic acid can reduce its cytoplasmic transfer and extracellular release by inhibiting HMGB1 phosphorylation. On the other hand, it can also directly inhibit the extracellular cytokine activity of HMGB1 and the activation of downstream inflammatory pathways, so as to alleviate neurotoxicity.

**Declarations**

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Figures
Figure 1

Figure 3-1 Effects of Glycyrrhizic acid and Semen Strychni on Nissl staining in the CA1 area of rat hippocampus. Representative photographs of Nissl staining obtained from the (A) Control group; (B) SS group; (C) SS + LGA group; (D) SS + HGA group.
Figure 2

Figure 3-2 Effects of Glycyrrhizic acid and Semen Strychni on Nissl staining in the CA1 and CA3 areas of rat hippocampus. Representative photographs of FJB staining obtained from the (A) and (E) Control group; (B) and (F) SS group; (C) and (G) SS + LGA group; (D) and (H) SS + HGA group.
Figure 3

Figure 3-3 Effects of Glycyrrhizic acid and Semen Strychni on TUNEL staining of rat cortex. Representative photographs of TUNEL staining obtained from the (A) Control group; (B) SS group; (C) SS + LGA group; (D) SS + HGA group.
Figure 3-4 The AChE (A) and MAO (B) levels of different group in the serum. Data were presented as means ± SEM (n = 8-10). *p < 0.05, **p < 0.01 vs. the control group. #p < 0.05##p < 0.01 vs. the SS group.
Figure 5

Figure 3-5 The HMGB1 levels of different group in the serum. Data were presented as means ± SEM (n = 8-10). *p < 0.05 vs. the control group. #p < 0.05 vs. the SS group.

Figure 6

Figure 3-6 Subcellular localization of HMGB1 in rat brain. Representative photographs of IF-HMGB1 obtained from the (A) Control group; (B) SS group; (C) SS + LGA group; (D) SS + HGA group.
Figure 7

Figure 3-7 Protein expression of HMGB1 in the nucleus and cytoplasm. A, Expression of HMGB1 protein in the nucleus. B, Expression of HMGB1 protein in the cytoplasm. The blot shown is representative of three experiments with similar results. Data were presented as means ± SEM (n = 3). *p < 0.05, **p < 0.01 vs. the control group. #p < 0.05, ##p < 0.01 vs. the SS group.
Figure 8

Figure 3-8 Effects of glycyrrhizic acid and Semen Strychni on levels of HMGB1 phosphorylation. A, Expression of phosphorylated HMGB1 protein in the nucleus. B, Expression of phosphorylated HMGB1 protein in the cytoplasm. Nuclear and cytosol extract were immunoprecipitated (IP) with an anti-HMGB1 antibody and then immunoblotted (IB) with an anti-p-Serine antibody and an anti-HMGB1 antibody. The blot shown is representative of three experiments with similar results. Data were presented as means ± SEM (n = 3). *p < 0.05, **p < 0.01 vs. the control group. #p < 0.05, ##p < 0.01 vs. the SS group.
Figure 9

Figure 3-9 Protein-protein interactions between HMGB1 and PP2A (A&B) and between HMGB1 and PKC (C&D) were examined by immunoprecipitation-linked immunoblotting. The whole cell lysates were prepared and immunoprecipitated with anti-HMGB1 antibody and then immunolotted with anti-PP2Ac antibody (A) or immunoprecipitated with anti-PP2Ac antibody and then immunolotted with anti-HMGB1 antibody (B). The whole tissue lysates were prepared and immunoprecipitated with anti-HMGB1 antibody and then immunolotted with anti-PKCα antibody (C) or immunoprecipitated with anti-PKCα antibody and then immunolotted with anti-HMGB1 antibody (D).
Figure 10

Figure 3-9 The TNF-α(A) and IL-1β(B) levels of different group in the serum. Data were presented as means ± SEM (n = 8-10). *p < 0.05, **p < 0.01 vs. the control group. #p < 0.05, ##p < 0.01 vs. the SS group.
Figure 11

Figure 3-10 Effects of glycyrrhizic acid and Semen Strychni on activation of MAPKs pathway. The blot shown is representative of three experiments with similar results. Data were presented as means ± SEM (n = 3). *p < 0.05, **p < 0.01 vs. the control group. #p < 0.05, ##p < 0.01 vs. the SS group.
Figure 12

Figure 3-11 Subcellular localization of NF-κB in rat brain. Representative photographs of IF NF-κB obtained from the (A) Control group; (B) SS group; (C) SS + LGA group; (D) SS + HGA group.