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Licorice Extracts Attenuate Nephrotoxicity Induced by Brucine Through Suppression of Mitochondria Apoptotic Pathway and STAT3 Activation*

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Summary: Licorice, one of the most widely used medicinal herbs in East Asia, has effects such as anti-inflammation, antioxidant, and detoxifying. This study aimed to evaluate the protective effect of licorice on brucine-induced nephrotoxicity. Sprague Dawley rats were administered with brucine intraperitoneally for 7 consecutive days with or without treatment with licorice. The content of blood urea nitrogen and creatinine in serum, the activities of superoxide dismutase and content of glutathione, malonaldehyde in kidney tissue were detected. Hematoxylin-eosin staining was employed to observe the histopathological changes of kidney. The expression and phosphorylation levels of protein were evaluated by Western blotting and immunohistochemical analysis. The results illustrated that treatment with licorice extracts (LE) significantly protected against the brucine-induced nephrotoxicity by reducing the content of blood urea nitrogen and serum creatinine, attenuating pathologic damage. The unbalance of oxidative stress was repaired by LE via increasing the level of glutathione, promoting the activities of superoxide dismutase and decreasing the content of malonaldehyde. In addition, LE overturned the influence of brucine on apoptosis-related protein and signal transducer and activator of transcription-3 (STAT3) activation. Taken together, these data demonstrate that licorice may attenuate brucine-induced nephrotoxicity via inactivation of oxidative stress and mitochondrial-mediated apoptosis pathway. More importantly, the renoprotective effects may be mediated, at least partly, by preventing the activation of STAT3 protein.
Key words: licorice; brucine; nephrotoxicity; apoptosis; STAT3

Brucine is a bitter alkaloid extracted from *Semen Strychnine*, which was widely used as a traditional Chinese herb medicine[1]. Its excellent effects such as anti-tumor[2], anti-inflammation and angiogenesis[3] had been attributed to brucine. It was noted that brucine inhibited the tumour cell proliferation and induced its apoptosis, accompanied with down-regulation of B-cell lymphoma-2 (Bcl-2) and up-regulation of Bcl-2 associated X protein (Bax) and caspase-3[4, 5]. But serious nephrotoxicity was reported in the experimental and clinical settings[6–8]. Earlier studies indicated that the concentrations of brucine in the kidney were much higher than those in the plasma and other tissues[9]. However, insufficient attention has been paid to the nephrotoxicity of brucine. The mechanism of renal injury caused by brucine remains unclear. Thus, high attention should be paid to systematical evaluation of the nephrotoxicity of brucine.

Licorice is the root of *Glycyrriza uralensis Fischer*, which has been used as a traditional medicine for centuries[10]. Mounting evidence showed that licorice had anti-inflammatory, antioxidant, and antimicrobial effects[11–13]. More importantly, it appears in more than half of traditional Chinese medicine prescriptions and plays important roles in moderating toxic effects or enhancing the activity of other herbs[14]. In particular, our previous studies have proved the interaction between brucine and licorice, indicating that licorice might have satisfying protection effects on brucine toxication[15]. The therapeutic roles of licorice in renal injury were also found in many studies[16–18]. Evidence revealed that licorice ameliorated cisplatin-induced nephrotoxicity through inactivation of p53 and up-regulation of nuclear factor E2-related protein (Nrf2)[19]. Moreover, licorice was certified to ameliorate gentamicin-induced nephrotoxicity and oxidative damage by scavenging oxygen free radicals, decreasing lipid peroxidation, and improving antioxidant defense[16]. In addition, licorice extracts

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(LE) were conducive to the diabetes nephropathy in rats, which may be through blocking protein kinase B (PKB) activation and transforming growth factor-β signaling[17, 18]. Experiments demonstrated that glycyrrhizic acid and 18β-glycyrrhetinic acid were the potential effective constituents[19–21]. Although renal toxicity induced by different drugs had various features and mechanisms[22], it is very promising that the renal injury caused by brucine can be alleviated by treatment with licorice. However, the underlying mechanism is still confused.

Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is a pleiotropic cascade essential to signal transduction of cytokines from the plasma membrane to the nucleus[23]. It has been described to mediate various cellular functions, including gene activation, cell differentiation, cell proliferation and apoptosis[24]. Evidence indicated that brucine was able to inhibit the proliferation of tumor cells via JAK/STAT signal pathway[25]. But, whether the nephrotoxicity of brucine was connected with JAK/STAT signal pathway has not been identified. Of all the JAK-STAT pathways, JAK2/STAT3 is fully studied and has been widely implicated in renal injury. Recent investigations indicated fluorofenidone protected against renal fibrosis by inhibiting STAT3 tyrosine phosphorylation[24]. JAK/STAT signaling also participated in protection against renal ischemia and reperfusion injury by dexmedetomidine[26]. Moreover, licorice constituents are the regulator of the JAK/STAT signal pathway[27].

In this study, we investigated the protective effects of licorice against brucine-induced nephrotoxicity in rats and identified the potential underlying molecular mechanism, which was supposed to be related to the JAK/STAT signal pathway.

1 MATERIALS AND METHODS

1.1 Chemicals

The dried roots of *Glycyrrhiza uralensis* were purchased from SanXiang Chinese Herbal Medicine Co. Ltd. (China, Lot: 20151021), authenticated by Professor Shao Liu (School of Pharmaceutical Sciences, Central South University, Changsha, China). The voucher specimens were deposited in the Herbarium, School of Pharmaceutical Sciences, Central South University, Hunan, China. Brucine was purchased from OnRoad Biotechnology Co. Ltd. (China). Anti-Bcl-2 antibody was obtained from Shenyang Wanlei Biological Technology Co. Ltd (China). Anti-cleaved-caspase-3, anti-cytochrome C, anti-phospho-STAT3 and anti-phospho-AKT were obtained from Cell Signaling Technology, Inc. (USA). Other antibodies such as anti-Bax, anti-caspase-3, anti-STAT3, anti-Fas, anti-caspase-8, anti-caspase-9, anti-GAPDH and anti-β-actin, were taken from Abcam Biotechnology Co. (UK). Kits for measurement of superoxide dismutase (SOD), malonaldehyde (MDA) and glutathione (GSH) were acquired from Nanjing Jiancheng Bioengineering Institute (China).

1.2 Preparation of Licorice Extracts

Licorice was soaked for 12 h and extracted with five-fold mass of water for 2 h in a reflux condenser twice. Then the decoctions were filtered, the filtrate was collected and concentrated. The final concentration of LE was 0.36 g of crude drug per milliliter. The contents of glycyrrhizic acid, glycyrrhetinic acid, liquiritigenin, isoliquiritigenin and liquiritin in LE were determined to be 3.55 mg/mL, 0.71 μg/mL, 0.17 mg/mL, 52 μg/mL and 1.05 mg/mL, respectively.

1.3 Animals

Male pathogen-free Sprague Dawley rats (220±20 g) were purchased from Hunan SJA Laboratory Animal Co. Ltd (China). The animals were acclimatized to a temperature-controlled environment (22±2°C) under a 12/12 h light/dark cycle for a week with free access to food and water. This experiment was approved by the Animal Care & Use Committee of Central South University. All the experiments were performed under the Guide for Care and Use of Laboratory Animals (Chinese Council).

The rats were randomly assigned to several groups (n=6) as follows: Control group (Control): Rats received vehicle. Brucine group (BR): Rats received brucine (50 mg/kg body weight) by intraperitoneal injection for 7 days. Licorice group (LE): Rats received LE (3.6 g/kg of crude drug per body weight) orally for 7 days. BR+LE group: Rats received brucine (50 mg/kg) and LE (3.6 g/kg) simultaneously for 7 days. The rats were anesthetized 24 h after drug administration, then the kidneys and plasma were collected. All the animals were euthanized by overdose of pentobarbital sodium at the end of the experiment.

1.4 Serum Biochemical Analysis

All plasma biochemical parameters including creatinine (Crea), blood urea nitrogen (BUN) were analyzed by an automatic biochemical analyzer (Roche, Rotkreuz, Switzerland) at the Second Xiangya Hospital of Central South University.

1.5 Antioxidant Activities

The extent of oxidative stress was estimated in kidney homogenates by measuring the activities of SOD and contents of GSH and MDA, using commercial kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China).

1.6 Histological Analysis

Renal samples were immersed in a 4% (w/v) paraformaldehyde solution for 24 h, then hydrated by graded alcohol, deparaffinized by xylene, and embedded in paraffin, cut into 4-μm-thick sections. The sections were stained by hematoxylin-eosin (HE).

The histomorphology abnormalities were detected by light microscopy.

1.7 Immunohistochemical Localization of Caspase-8 and Caspase-9

Paraffin-embedded sections were deparaffinized, washed with phosphate buffered saline (PBS), blocked by 3% (v/v) H_2O_2, incubated with 10% (w/v) normal horse serum for 1 h, then immersed in primary antibody at 4°C overnight. Anti-caspase-8 and anti-caspase-9 were the primary antibodies for detection of caspase-8 and caspase-9 (Abcam Biotechnology Co., UK). Then the slides were incubated with biotinylated secondary antibodies (1:1000) for 20 min. Finally, the immunostaining was visualized using diaminobenzidine reagent.

1.8 Western Blotting

Protein samples were prepared by homogenization with ice-cold PBS and lysed in radio immunoprecipitation assay lysis buffer (Cwbiotech, China). Protein samples underwent sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) and were transferred to polyvinylidene fluoride membrane. After being blocked in 5% non-fat milk for 1 h at room temperature, the membranes were incubated with primary antibodies, which included anti-Bcl-2, anti-Bax, anti-caspase-3, anti-cleaved-caspase-3, anti-phospho-AKT, anti-STAT3, anti-Fas, anti-cytochrome C, anti-phospho-STAT3, anti-GAPDH and anti-β-actin, and stored overnight at 4°C. Subsequently, the immunoblots were incubated with the secondary antibody at room temperature for 2 h. The membranes were tested using Pierce™ enhance chemiluminescence (ECL) Western Blotting Substrate according to the manufacturer's instructions (Thermo Fisher Scientific, USA) .

1.9 Statistical Analysis

Data were presented as mean±standard deviation (SD). SPSS software (version 18.0) was used for statistical analysis. All comparisons were analyzed by one-way analysis of variance, followed by Turkey's post hoc test. A value of $P<0.05$ was considered significant.

2 RESULTS

2.1 Licorice Treatment Improved Renal Function

To investigate the effect of LE on brucine-induced nephrotoxicity, levels of serum Crea and BUN were measured (fig. 1). Brucine increased the levels of Crea and BUN, indicating that brucine resulted in severe nephrotoxicity. LE attenuated the increase of Crea and BUN significantly. LE alone had no notable effect on serum Crea and BUN compared with the control group.

2.2 Effect of Licorice on Brucine-induced Oxidative Stress in Rats

Compared with the control group, the renal GSH level and the activities of SOD were significantly reduced while MDA content was significantly increased in brucine-treated group (fig. 2). LE ameliorated the renal oxidative stress by raising the level of GSH, promoting the activities of SOD and reducing the content of MDA. Besides, there were no significant differences in the renal GSH, MDA and SOD levels between LE group and control group. However, there were significant differences between the control group and LE detoxification groups.

2.3 Licorice Treatment Attenuated Histological Lesion in Brucine-induced Rats

After 7 days treatment with brucine, abnormal histological features were observed. The renal histology from brucine-treated rats showed severe pathological changes such as edema of glomerular epithelial cells, inflammatory cell infiltration of the glomerulus and partial necrosis of glomeruli (fig. 3C). In the LE group, the degree of pathological changes was significantly decreased. Inflammatory cell infiltration and cell necrosis were rarely found. However, edema was discovered in renal tubular epithelial cells (fig. 3D). The morphological structure in the control group was normal (fig. 3A).

2.4 Influence of Licorice on Renal Expression of Caspase-8 and Caspase-9

The immunohistochemistry image and mean absorbance value of caspase-8 and caspase-9 are

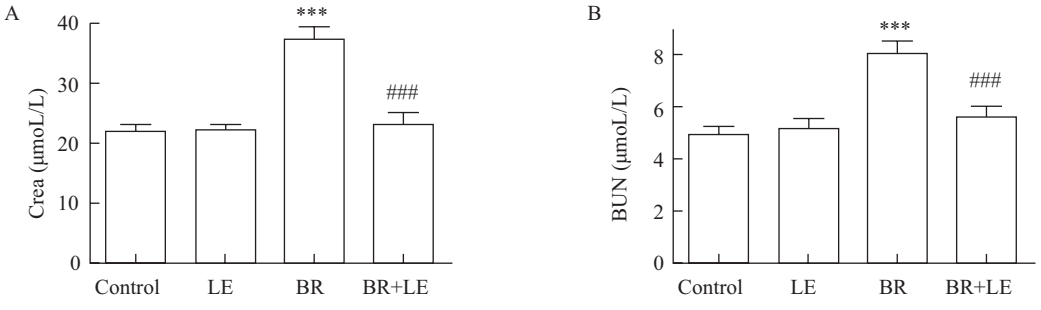

Fig. 1 Effects of LE on alterations of renal function following brucine (BR) induced renal injury
Serum Crea (A) and BUN (B) were measured to assess the renoprotective effect of licorice against brucine-induced renal injury (n=6, mean±SD). $^{***}P<0.001$ *vs.* the control group; $^{\#\#\#}P<0.001$ *vs.* the BR group

shown in fig. 4 and 5. Brucine increased the expression of caspase-8 and caspase-9 in the renal tubule (fig. 4C and 5C). In the rats treated with LE, the expression level of caspase-9 decreased significantly. However, LE had no obvious effect on caspase-8.

2.5 Effects of Licorice on Expression of Apoptotic Proteins in Brucine-induced Rats

In this study, several apoptosis-associated proteins including Bcl-2, Bax, caspase-3, cleaved-caspase-3, Fas and cytochrome C were measured. The representative photographs and the quantitative analysis of protein expression are shown in fig. 6. Brucine elevated the expression levels of Bax, cytochrome C, caspase-3 and cleved-caspase-3 proteins. In the meantime, the expression of Bcl-2 protein and ratio of Bcl-2/Bax were diminished by brucine treatment. Compared with BR group, LE treatment decreased the expression levels of Bax, cytochrome C, caspase-3 and cleaved-caspase-3 greatly, but increased the expression of Bcl-2 and ratio of Bcl-2/Bax. Unexpectedly, Western blotting indicated that Fas expression was unaffected after brucine or/and licorice treatment.

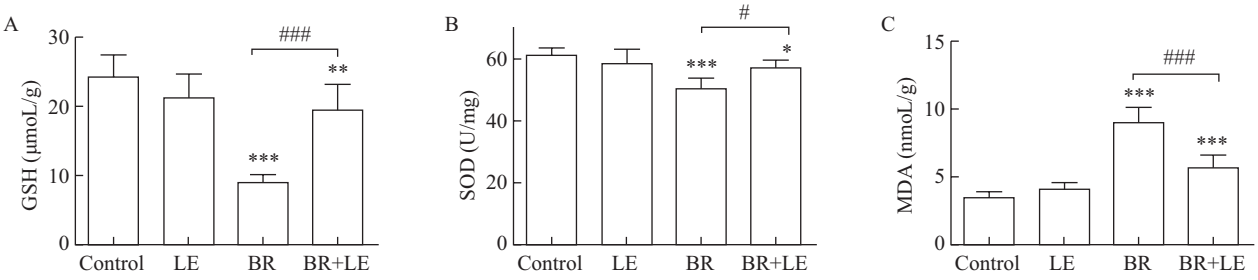

Fig. 2 LE inhibited brucine (BR)-induced oxidative stress
A: The level of GSH in kidney was detected. B: The activities of SOD were measured. C: The content of MDA was determined. Each value represents the mean±SD (n=6). $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 *vs.* the control group; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001

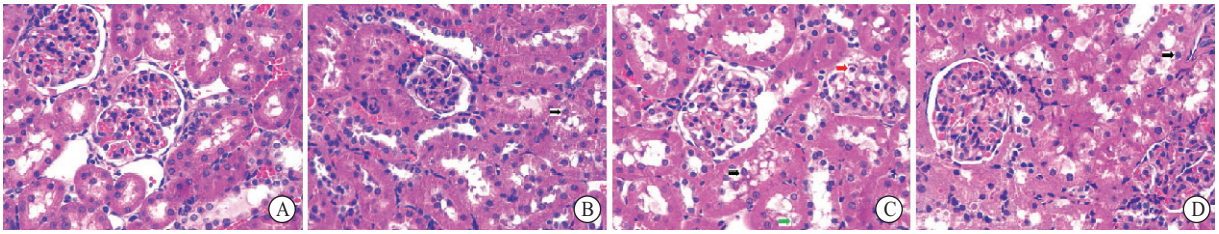

Fig. 3 Histological analysis of kidney tissues from rats in control (A), LE (B), BR (C) and BR+LE (D) groups
Tissue sections were H&E stained (×400). Different kinds of pathology lesions were marked by different arrows. The black arrows meant cellular edema, the red arrows represented inflammatory cell infiltration, and the green arrows indicated cell detachment.

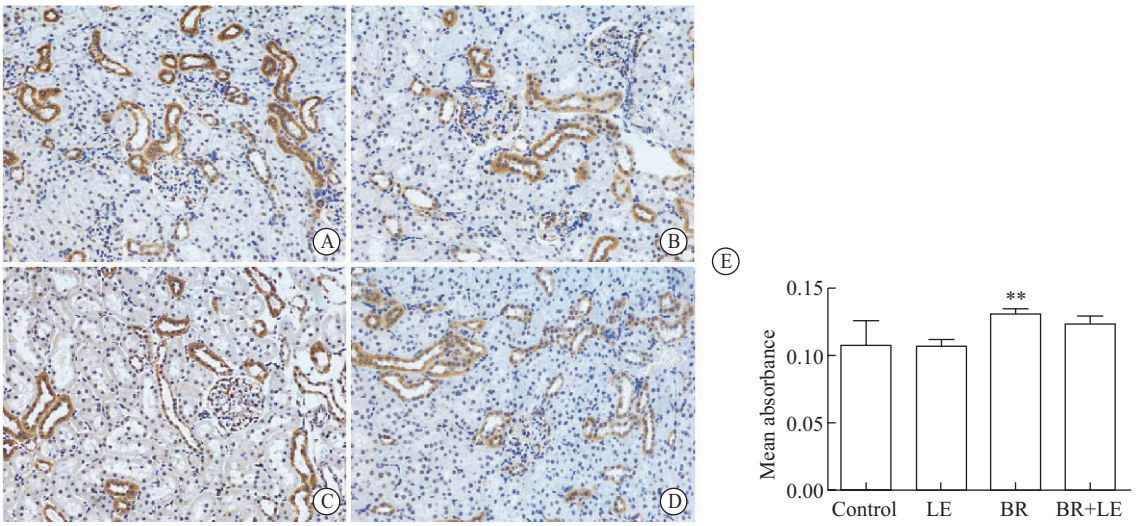

Fig. 4 The effect of LE on expression of caspase-8 in the kidneys (×200)
Immunohistochemical staining of caspase-8 was performed on formalin-fixed paraffin-embedded kidneys of the Control (A), LE (B), BR (C) and BR+LE (D) groups. E: semi-quantitative assessment of caspase-8 expression based on mean absorbance value. Data were represented as mean±SD (n=5). $^{**}P$<0.01 *vs.* the control group

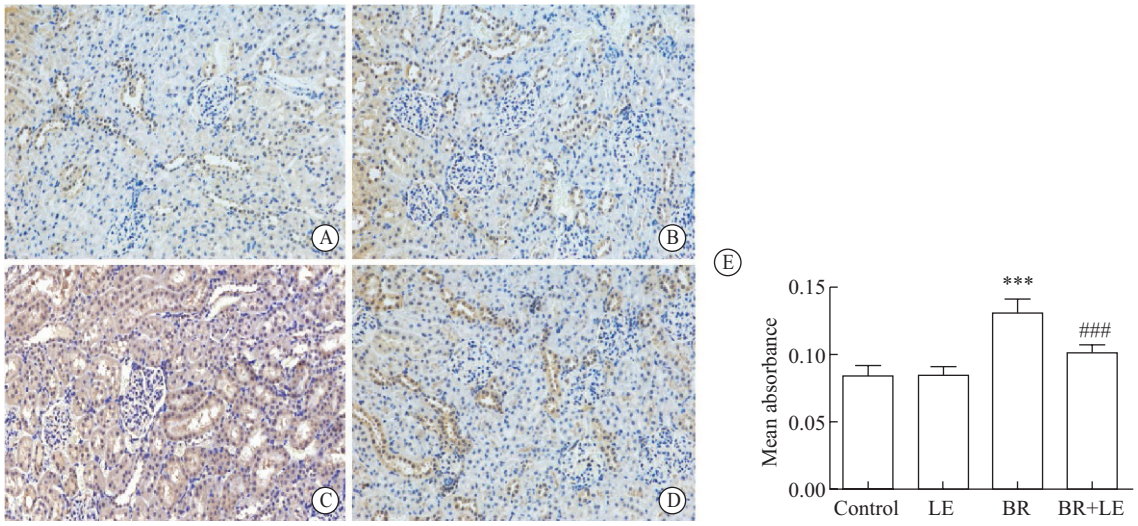

Fig. 5 Effect of licorice on expression of caspase-9 in the kidneys (×200)
Immunohistochemical staining of caspase-9 was performed on formalin-fixed paraffin-embedded kidney tissues of the Control (A), LE (B), BR (C) and BR+LE (D) groups. E: semi-quantitative assessment of caspase-9 expression based on mean absorbance value. Data were represented as mean±SD (n=5). $^{***}P$<0.001 *vs.* the Control group, $^{###}P$<0.001 *vs.* the BR group

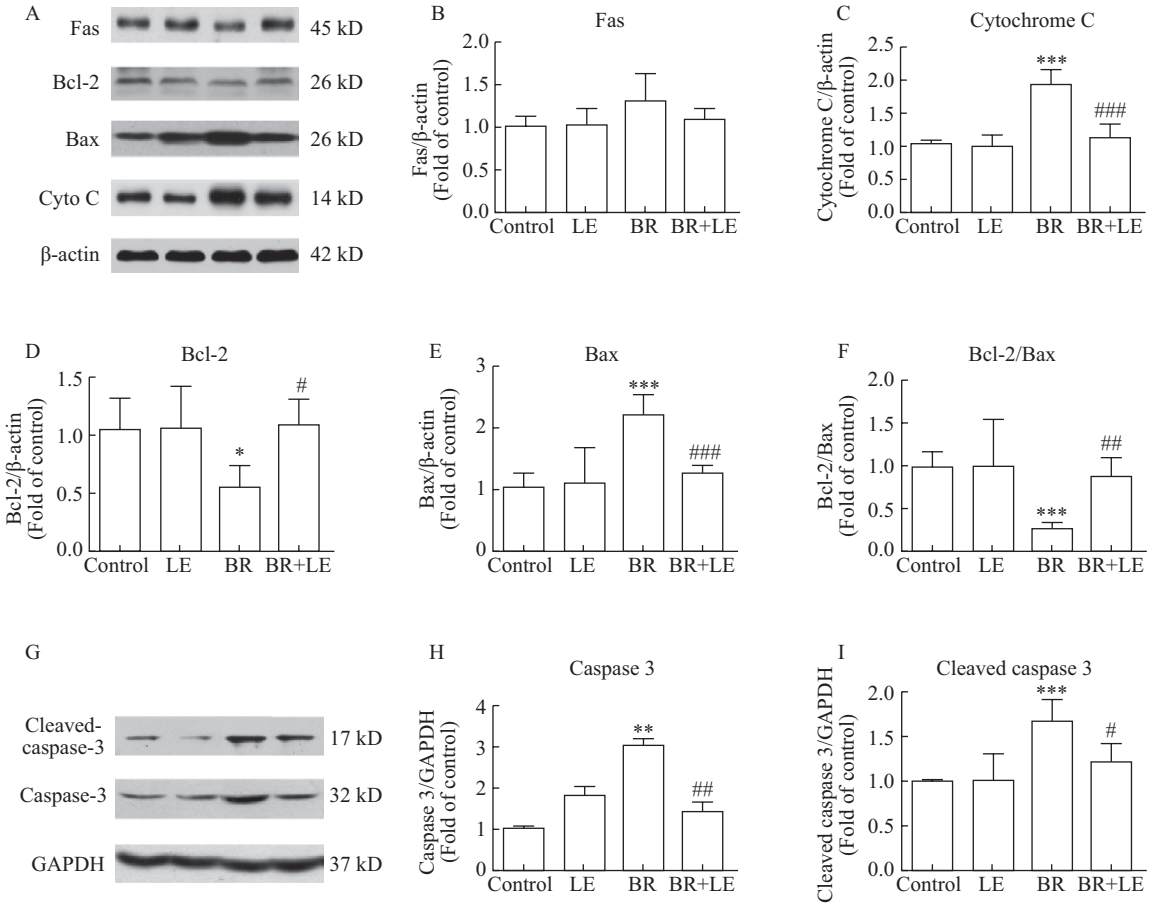

Fig. 6 LE inhibited the apoptosis proteins induced by brucine (BR) in renal tissues.
A: Expression levels of Bax, Fas, Bcl-2, cytochrome C (Cyto C) in renal tissue were assessed by Western blotting. Representative immunoblots of the indicated proteins are shown. Protein levels of Fas (B), Cytochrome C (C), Bcl-2 (D), Bax (E), Bcl-2/Bax (F) were quantified and normalized to β-actin. G: The expressions of caspase 3, cleaved caspase 3 in renal tissues were measured by Western blotting. Protein levels of caspase 3 (H) and cleaved caspase 3 (I) were quantified and normalized to GAPDH. Data were represented as mean±SD (n=5). $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 *vs.* the Control group; $^{#}P$<0.05, $^{##}P$<0.01, $^{###}P$<0.001 *vs.* the BR group

2.6 Licorice Inhibited Activation of Renal p-STAT3 Protein

STAT3 and p-STAT3 proteins were mainly expressed in renal tubular epithelial cells and stromal vascular endothelial cells[28]. Normal rat kidneys had weak expression of STAT3 and p-STAT3 proteins. The expression of p-STAT3 and p-STAT3/STAT3 significantly increased in kidneys of the BR group (fig. 7). Licorice treatment abolished the effects on the activation of p-STAT3 protein induced by brucine.

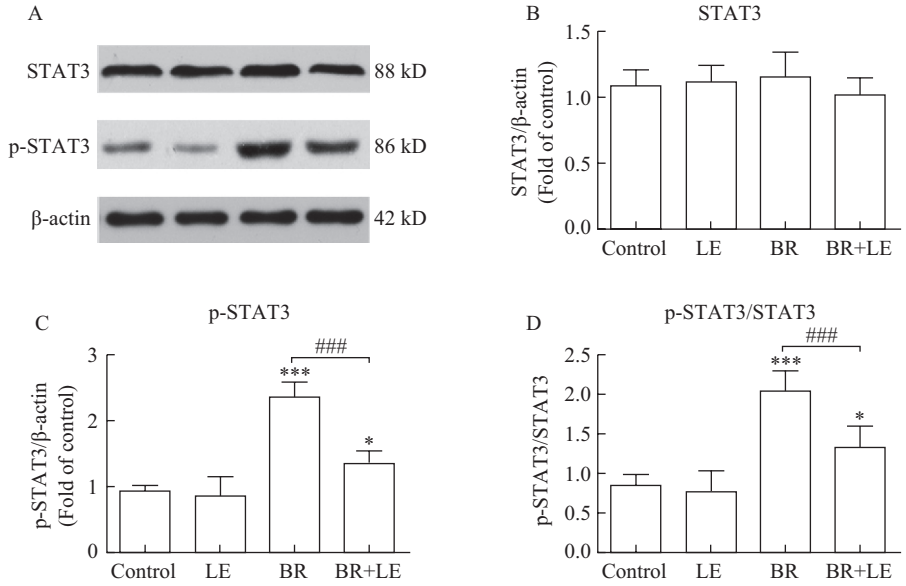

Fig. 7 LE suppressed the activation of STAT3 induced by brucine (BR)
Representative Western blots for the STAT3 and p-STAT3 expression (A) of the kidneys were detected. Protein levels of STAT3 (B) and p-STAT3 (C) were quantified and normalized to β-actin. D: Densitometry analysis of Western blots for the ratio of p-STAT3/STAT3. Data are represented as mean±SD (n=5). $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 *vs.* the Control group; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001

3 DISCUSSION

Numerous studies had reported that licorice showed positive effects against drug-induced renal injuries. Moreover, several studies confirmed that licorice and glycyrrhizic acid could substantially ameliorate renal function by suppressing inflammation, apoptosis and oxidative stress via several signaling pathways[19, 29, 30]. Nevertheless, the effect of licorice on brucine-induced kidney injury and its related molecular mechanism were poorly understood. In the present study, we verified that brucine promoted apoptosis in the rat kidney after 7-day treatment, and the apoptosis was mediated via mitochondrial apoptosis pathway. In a word, licorice ameliorated brucine-induced nephrotoxicity through inhibiting apoptosis and oxidative stress via suppressing the p-STAT3 signaling activation (fig. 8).

Nephrotoxicity is one of the main side effects that limit the therapeutic efficacy of brucine[31]. Renal failure caused by semen strychnine could be ameliorated by *Radix Glycyrrhizae* and *Rhizoma Ligustici*[32]. Serum Crea and BUN levels were important clinical indexes that could reflex the condition of kidney[33]. Histopathological sections can help us understand the histopathological changes directly. Our results showed that serum Crea and BUN in BR group were significantly increased,

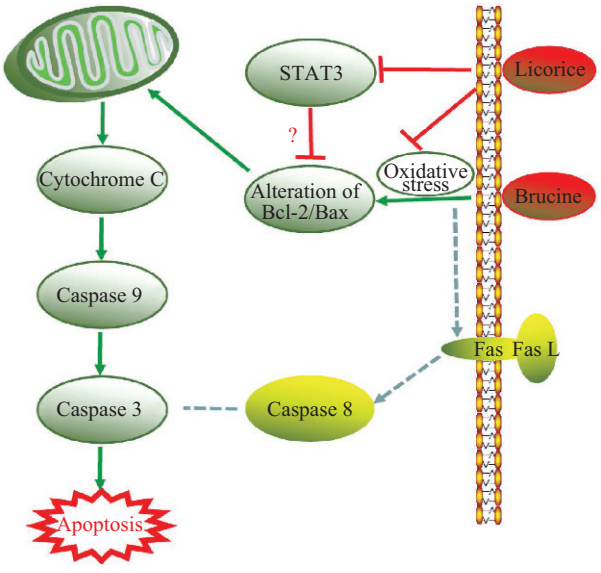

Fig. 8 Schematic diagram of brucine-induced nephrotoxicity and its improvement by licorice

which consist with the histopathological changes. These results might indicate that rats in brucine group showed serious renal injury. The levels of serum Crea, BUN and histopathological characteristics in the BR+LE group were similar to those of the control group. The results

indicated that licorice could partly attenuate the renal injury caused by brucine.

We also found that the protective effect of licorice against brucine-induced renal cell damage was associated with the prevention of oxidative stress, which implied that the antioxidant properties of licorice might play an important role in protecting cells from brucine-induced apoptosis. The antioxidant enzymes SOD and free radical scavenger GSH play major roles in maintaining cellular redox homeostasis[34]. A decrease in SOD activity and GSH concentration reduced cellular removal of hydrogen peroxide and superoxide ion radicals, leading to increased level of MDA[35]. In our research, brucine exposure decreased the activities of SOD and contents of GSH meanwhile increasing the levels of MDA. Co-administration with licorice induced a significant increase in renal GSH contents and SOD activities, along with a decrement in MDA levels. These results are in accordance with several researches highlighting the antioxidant properties of licorice *in vivo* and *in vitro*[20, 36, 37]. The oxidant/antioxidant imbalance in the normal redox status of cell can cause an increment in reactive oxygen species (ROS). Excessive ROS induced cell death by stimulating the intrinsic apoptotic pathway[38].

Apoptosis, programmed cell death, can be induced by both the death receptor and mitochondrial pathways[39, 40]. The first pathway involves death receptors and their ligands, resulting in the recruitment of adaptor proteins and procaspase molecules. The complex is an apoptosome in which the aggregated procaspase transactivates. The active caspases (e.g., caspase-8) then responds to cleave and activate the downstream caspases[39]. Several lines of evidence indicated that licorice constituents could modulate cell apoptosis via Fas/Fas ligand[41, 42]. Things were different in our results which suggested that Fas and its downstream caspase-8 were not involved in the protection of licorice against brucine-induced nephrotoxicity. The possible reason was that liquiritin and licochalcone A may be the active ingredients for death receptor, but those two ingredients had low content in LE.

Studies have shown that the apoptotic effects of brucine were mediated via Bcl-2 and Ca^{2+} involved mitochondrial pathway[2]. The mitochondrial pathway of cell death is mediated by Bcl-2 family proteins. Among them, the apoptosis-promoting protein Bax and the anti-apoptosis protein Bcl-2 are two important proteins. The ratio of Bcl-2/Bax unbalance disrupts the mitochondria membrane potential and results in release of cytochrome C into the cytosol[40]. Cytochrome C then forms an apoptosome containing caspase-9, which then activates the downstream apoptotic signals[40]. Our results reinforced the importance of mitochondria apoptotic pathway in brucine-induced nephrotoxicity. Evidence has illuminated that licorice compounds were potent modulators of mitochondrial dysfunction in hepatocytes and neuronal cells[43–45]. However, whether licorice protected brucine-induced nephrotoxicity and the underlying critical role of mitochondria apoptosis pathway were still unknown. Our data suggested that LE could decrease the expression of mitochondria apoptosis regulated proteins such as Bax, Cytochrome C, caspase-9 and caspase-3, but increased the expression of Bcl-2. Most importantly, this study has unexpectedly revealed that STAT3 was a mechanistic target of licorice in renal protection, through which licorice protected against mitochondrial apoptosis induced by brucine.

STAT3 is localized in tubular epithelium and widely involved in sensing cellular stress, inflammatory response and immune regulation[46, 47]. Several recent studies mentioned that JAK/STAT3 cascade has been widely implicated in the pathogenesis of nephropathy. In detail, Koike indicated that JAK/STAT3 pathway might exert protective effect against renal fibrosis in unilateral ureteral obstruction model[48]. Moreover, Lu[49] illustrated that STAT3 played a crucial role in the modulation of inflammation and abnormal matrix synthesis by using STAT3 knockdown rats with streptozocin-induced diabetes. Our results revealed that STAT3 was activated in rats treated with brucine, subsequently mediated cell apoptotic signals through the Bcl-2 and caspase-3 apoptin. To our knowledge, this is the first report illuminating the molecular mechanism in brucine-induced nephrotoxicity. Although, brucine was already confirmed to induce apoptosis of several kinds of tumour cells via mitochondrial pathway[2, 25]. Furthermore, we have provided evidence that licorice might protect the kidneys via the STAT3 pathway and preventing mitochondrial apoptosis pathway. The same as JAK/STAT signaling, phosphatidylinositol-3 kinase/serine-threonine kinase B (PI3K/Akt) signal pathway plays an essential role in promoting and modulating stress and apoptosis processes[50]. Numerous studies also inferred that many pharmaceutical or nutraceutical products (for example, licorice) with potent antioxidant and anti-apoptosis activity can regulate various signaling molecules in PI3K/Akt pathway, supporting its renoprotective activity[51–54]. Thus, we evaluated the p-AKT expression, but the consequence was negative. The possible reason was that PI3K/Ak was not involved in brucine-induced nephrotoxicity.

In summary, our results suggested that licorice attenuated renal injury induced by brucine through regulation of oxidative stress and mitochondrial-mediated apoptosis pathway. More importantly, the renoprotective effects may be mediated, at least partly, by preventing the activation of STAT3 protein.

Conflict of Interest Statement

The authors declare no competing interests.

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