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The study of the *Oxytropis kansuensis*-induced apoptotic pathway in the cerebrum of SD rats

Hao Lu, Liang Zhang, Shan-shan Wang, Wen-long Wang and Bao-yu Zhao*

Abstract

Background: Locoweeds cause significant livestock poisoning and economic loss all over the world. Animals can develop locoism, a chronic neurological disease, after grazing on locoweeds. *Oxytropis kansuensis* is a variety of locoweed that contains swainsonine as its main toxic ingredient. The purpose of this study was to investigate the apoptotic pathway induced in the cerebrum by swainsonine.

Results: Twenty-four Sprague-Dawley rats were randomly divided into four groups (experimental groups I, II, III and a control group) and 6 SD rats of each group were feed in 3 cages separately. Rats were penned as groups and fed with feeds containing 15% (SW content 0.03‰), 30% (SW content 0.06‰), or 45% (SW content 0.09‰) *O. kansuensis* for experimental groups I, II, and III, respectively, or complete feed in the case of the control group. One hundred and nineteen days after poisoning, and all rats showed neurological disorders at different degrees, which were considered to be successful established a chronic poisoning model of *O. kansuensis*. rats were sacrificed and the expression of Fas, FasL, Bcl-2, Bax as well as cleaved caspase-3, -8 and -9 proteins in brain tissues were detected by Western blot. The results showed that SW treatment up-regulated Fas and Fas ligand (FasL) ($P < 0.05$), and that there was an increase in Bax and a decrease in Bcl-2 protein ($P < 0.01$). Moreover, SW treatment significantly increases the activation of caspase-3, 8 and -9, the key effectors in apoptosis pathway ($P < 0.01$).

Conclusion: Our data suggest that SW induces apoptosis in cells of the brain through death receptor and mitochondria-mediated, caspase-dependent apoptotic pathways in the brain tissue of SD rats.

Keywords: *Oxytropis kansuensis*, Swainsonine, SD rats, Cerebrum, Apoptotic pathway

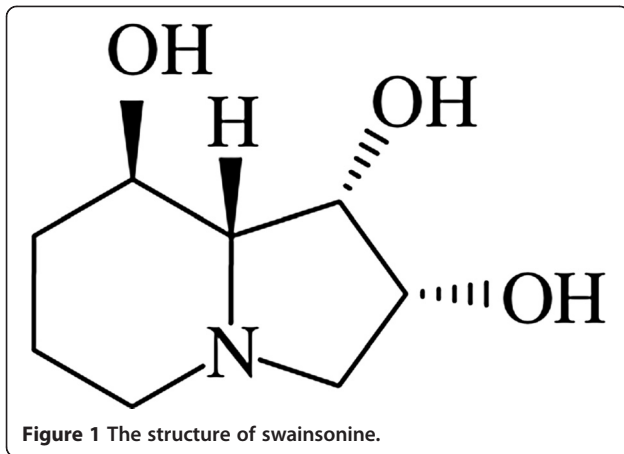
Background

Locoweeds are a general term for toxic plants of the genera *Astragalus* and *Oxytropis*, and are one of the toxic plants causing the most damage to husbandry in the grassland across the world. They possess wider geographical distribution from the Great Plains to the Rocky Mountains, and they are worldwide spread, such as in the United States [1], Australia [2], Mexico [3], Brazil [4], and China [5]. *Oxytropis kansuensis*, one of the locoweeds from the genus *Oxytropis*, is found in Qinghai, Gansu, Ningxia, Sichuan, northwestern Yunnan, and paramos regions in Tibet [6]. Because of its luxuriant leaves, wide-spread roots and strong stress resistance, huge numbers of livestock get poisoned and even die in grassland containing *Oxytropis kansuensis* every year, causing huge losses to local herdsmen and typical

ecological-economic disease [7]. Poisoned animals show clinical signs characterized by nerve functional disturbance symptoms like depression, diminished response, ataxia, abnormal behavior, emaciation and decline in immune function [8,9].

The main toxic ingredient in locoweeds is swainsonine (SW) [10], an indolizidine alkaloid whose chemical name is 1, 2, 8- trihydroxy-indolizidine alkaloid (Figure 1). SW was first isolated from *Swainsona canescens* by Colegate et al. [11]; since then, large amounts of researches has focused on the bioactivity of SW, finding that the structure of SW is similar to the mannose cation formed by the hydrolysis of mannosidase. SW has high affinity to α -mannosidase and, as a result, can inhibit the enzyme. This inhibition induces abnormal of glycoprotein processing and cellular vacuolation [12], especially in nerve cells that is caused by the accumulation of oligosaccharides in the lysosome [13,14].

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Apoptosis is a tightly controlled physiological process that plays a critical role in developmental modeling, homeostasis maintenance, immune repertoires, and clearance of infected or transformed cells [15]. Apoptosis can be triggered by various extracellular and intracellular stimuli via either an extrinsic or intrinsic pathway in different cells [16]. The extrinsic pathway is initiated by cell surface receptors, while the intrinsic pathway is initiated by a mitochondria mediated death signaling cascade [16]. To date, SW has been reported to induce the apoptosis of the gastric cancer cell SGC-7901, C6 glioma cells, human lung cancer cell A549, etc. Some studies have been conducted examining the apoptosis pathway induced by SW in A549 cells [17-19]. However, the mechanism SW-mediated neurotoxicity has not been rigorously explored, and apoptosis in the brain induced by SW and its associated pathways have yet to be

discovered. Thus, the objective of this study was to determine the effects of SW on the expression of Fas, FasL, Bcl-2, Bax and cleaved caspase-3, -8 and -9 in the brains of SD rats and to determine which apoptosis pathway is induced by SW. These results will inform future research on the mechanisms underlying the toxicity of *O. kansuensis* as well as other locoweeds.

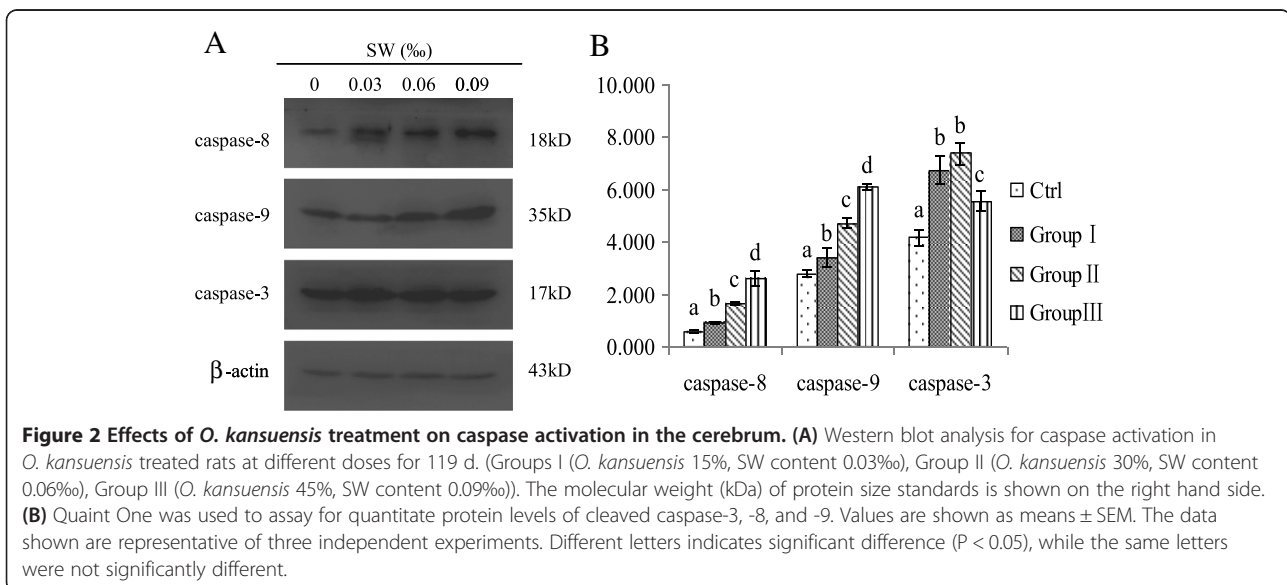
Results

O. kansuensis activates caspase-8, -9 and -3

To gain insight into the underlying mechanism of *O. kansuensis*-induced apoptosis, we measured the activation of caspases in the brain cells of these rats. Protein levels of caspase-8, -9 and -3 were measured by Western blot. Upon *O. kansuensis* treatment, cleaved caspase-8, cleaved caspase-9 and cleaved caspase-3 levels increased with increased toxic doses, cleaved caspase-8 and cleaved caspase-9 showed a dose-dependent increase (Figure 2). These data suggest that both death receptor pathway and mitochondrial pathways may contribute to caspase-3 activation in *O. kansuensis*-induced apoptosis.

O. kansuensis induces apoptosis through the Fas/FasL-dependent pathway

Caspase-8 is the initiator caspase for the caspase cascades activated by death receptor pathways [20]. The activation of caspase-8 suggested that Fas and FasL may be involved in *O. kansuensis*-induced apoptosis. Therefore, we investigated the expression of Fas and FasL in the brain cells of SD rats treated with *O. kansuensis*. As shown in Figure 3, the protein levels of Fas and FasL were increased in a dose-dependent manner.



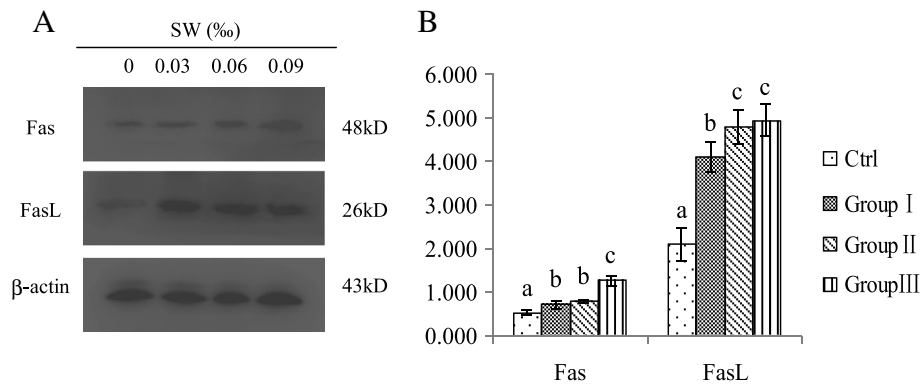


Figure 3 Effect of *O. kansuensis* treatment on Fas and FasL expression in the cerebrum. (A) SD rats were treated with *O. kansuensis* at different doses for 119 d (Groups I (*O. kansuensis* 15%, SW content 0.03%), Group II (*O. kansuensis* 30%, SW content 0.06%), Group III (*O. kansuensis* 45%, SW content 0.09%)); β -actin was used as an internal loading control. Protein levels of Fas and FasL were analyzed by Western blot. The molecular weight (kDa) of protein size standards is shown on the right hand side. (B) Quaint One was used to quantitate protein levels of Fas and FasL. The results are mean \pm SD and representative of three independent experiments. Different letters indicates a significant difference ($P < 0.05$), while the same letters were not significantly different.

O. kansuensis regulates the expression of Bcl-2 family proteins

The expression of Bcl-2 and Bax were detected by Western blot. Protein levels of Bax were up-regulated, while, conversely, the protein levels of Bcl-2 were significantly decreased (Figure 4A). As shown in Figure 4B, *O. kansuensis* resulted in a dose-dependent increase in the ratio of Bax/Bcl-2. These results suggested that Bax up-regulation and Bcl-2 down-regulation may play important roles in *O. kansuensis*-induced apoptosis of brain cells in SD rats.

Discussion

The indolizidin alkaloid SW is present in *O. kansuensis*, and has been shown to have neurotoxic effects. Previous studies have reported that the poisoning of livestock by locoweeds caused neurons in the cerebral cortex, basal ganglia, thalamus, mid-brain, hippocampus, cerebellum, medulla, and spinal cord to undergo cytoplasmic vacuolar degeneration [21]. Colodel et al. reported that *Swainsona*, *Oxytropis*, *Astragalus*, and *Ipomoea* poisonings caused multiple cytoplasmic vacuoles in acinar pancreatic cells, hepatocytes, and renal tubular cells, especially in neurons, and led to lesions of the central nervous system [22]. In the present study, we investigated the mechanisms of *O. kansuensis*-induced apoptosis of brain cells in SD rats. These data demonstrated that SW induced apoptosis in the cerebrum via the Fas/FasL and mitochondria-mediated, caspase-dependent apoptotic pathways.

Many previous reports have indicated that numerous neurogenic diseases were related to the apoptosis of neurons [23-25]. Sun et al. reported that SW could induce SGC-7901 cell apoptosis by inhibiting the gene p53 and decreasing the expression of Bcl-2, increasing the apoptotic trigger gene c-myc and loading $[Ca^{2+}]_i$ [17]; they

subsequently demonstrated that the mechanisms of SW-induced apoptosis may be related to the expression of apoptosis-related genes and overloading- $[Ca^{2+}]_i$ -induced endoplasmic reticulum stress [18]. Li et al. reported that SW treatment up-regulated Bax, down-regulated Bcl-2 expression, increased the rate of Bax/Bcl-2, and activated the mitochondria-mediated, caspase-dependent apoptotic pathway *in vitro* and *in vivo*. A549 cells apoptosis occurred in a concentration- and time-dependent manner [19].

Apoptosis programs a cell to actively commit suicide as the results of activation of dedicated intracellular program; the death receptors (extrinsic) and mitochondrial (intrinsic) pathways are the major signaling cascades that lead to apoptosis. The death receptor pathway involves death receptors from the tumor necrosis factor receptor family such as Fas (CD95), TNF α R, DR3, DR4 and DR5. In the death receptors pathway, ligands of the death receptor initiate signaling via receptor oligomerization, which results in the recruitment of specialized adaptor proteins and the activation of caspase-8 [26]. The mitochondrial pathway is dependent on the formation of lipidic pores, which significantly affected the release of cytochrome *c* from the inner membrane of the mitochondria to the cytosol. Holocytochrome *c* induces Apaf-1 oligomerization, leading to the activation of caspase-9. Caspase-8 and -9 are both initiator caspases and activate downstream effector caspases that are essential for the direct demolition of cellular structures and DNA fragmentation associated with apoptosis [27-29].

Caspases involved in apoptosis can be divided into two functional subgroups based on their roles. Initiators (caspase-2, -8, -9 and -10) are responsible for initiating the activation of caspase cascades for different apoptotic

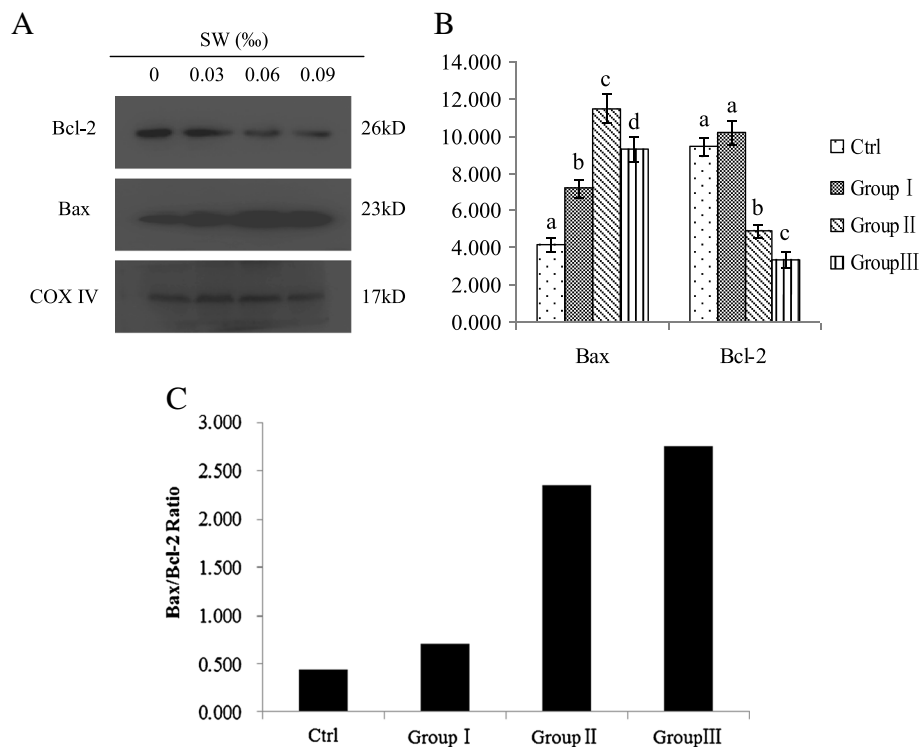


Figure 4 Effect of *O. kansuensis* treatment on the expression of Bcl-2 family proteins in the cerebrum. **(A)** SD rats were treated with *O. kansuensis* at different doses for 119 d (Groups I (*O. kansuensis* 15%, SW content 0.03%), Group II (*O. kansuensis* 30%, SW content 0.06%), Group III (*O. kansuensis* 45%, SW content 0.09%). Protein levels of Bcl-2 and Bax were analyzed by Western blot. The molecular weight (kDa) of protein size standards is shown on the right hand side. **(B)** Quaint One was used to quantitate protein levels of Bcl-2 and Bax. Values are shown as means \pm SEM, The data shown are representative of three independent experiments. Different letters indicates a significant difference ($P < 0.05$), while the same letters were not significantly different. **(C)** Data from **(A, B)** was used to evaluate the effect of *O. kansuensis* on the ratio of Bax/Bcl-2.

pathways. Effector caspases (caspase-3, -6 and -7) are responsible for demolition of the cell during apoptosis [20]. In this study, we observed that SW-induced apoptosis activated caspase-8, -9 and -3, which suggested that the death receptor-mediated caspase-8 pathway and the mitochondrial-mediated caspase-9 pathway may be responsible for SW-induced apoptosis.

Fas is one of the death receptors; binding of FasL induces Fas trimerization, which recruits caspase-8 via the adaptor protein Fas-associated death domain protein (FADD). Then, caspase-8 oligomerizes and is activated through autocatalysis. Activated caspase-8 triggers the execution phase of apoptosis via the activation of the downstream effector caspase-3 [20,26,30-32]. This study revealed that the SW-induced apoptosis resulted in increased expression of Fas and FasL, followed by activation of caspase-8 and -3. These data demonstrated that caspase-8 activation in SW-induced apoptosis can be mediated by Fas/FasL interaction.

Cytochrome *c* is one of a host of pro-death molecules residing within mitochondria and is a universal feature of apoptosis. Previous studies have demonstrated that

the Bcl-2 family is intimately involved in the regulation of cytochrome *c* release into the cytosol [33]. Bcl-2 family proteins include both pro- and anti-apoptotic members. The pro-apoptotic homolog Bax is located in the cytosol, and it can interact with the anti-apoptotic protein Bcl-2. In response to apoptotic signals, Bax translocates to the mitochondria and inserts into the outer mitochondrial membrane, heterodimerizing with Bcl-2 to abrogate Bcl-2's inhibition of apoptosis by promoting the release of cytochrome *c* into cytosol [34]. Therefore, the ratio of Bax/Bcl-2 sets the threshold of susceptibility to apoptosis for the mitochondrial pathway [20,31,32,35]. Our results showed that SW treatment up-regulated Bax and down-regulated Bcl-2 expression, increasing the ratio of Bax/Bcl-2 which likely resulted in brain cell apoptosis.

Conclusions

Our results demonstrate that SW induces apoptosis through the activation of Fas/FasL-mediated caspase-8 and mitochondria-dependent caspase-9 pathways. These data provided a new foundation to further explore the underlying mechanisms of *O. kansuensis*-induced apoptosis.

Based on these findings, a schematic model of possible SW-induced apoptotic mechanisms in brain cells is shown in Figure 5. However, it is probable that we are far from unraveling the complete mechanisms of SW-induced apoptosis, with mechanisms involving p53, AIF, Smac/DIABLO, and endoplasmic reticulum stress potentially playing a significant role.

Methods

Plant materials

The aerial portion of *O. kansuensis* was collected in Huangzhong County (36°29.286'N; 101°41.499'E), Qinghai Province in August 2008. The plants were preserved in a shady place after air-drying and comminution. SW was extracted by TCL and GC-MS methods [36], SW content was determined by gas chromatography as 0.021% of the total plants content. The plant was identified by the Institute of Botany (Life Science College, Northwest A&F University).

Animals and animal feed

The animals and protocols used in this study were approved by the Animal Care Committee of Xi'an Jiaotong University. SD rats were purchased from the Experimental Animal Center of College of Medicine, Xi'an Jiaotong University (Xi'an, China). The aerial portion of *O. kansuensis* was completely ground, then sifted through a 200 mesh

screen. The resulting *O. kansuensis* grass meal was mixed with whole feed (25% flour, 28% cornmeal, 20% soya-bean cake, 10% wheat bran, 10% fish meal, 1% vegetable oil, 1% yeast powder, 2% bone meal, 1% cooking salt, 1% cod-liver oil, 0.9% mineral additive, 0.1% vitamin additive) [37] to obtain 15%, 30%, and 45% grass content feed containing 0.03%, 0.06%, and 0.09% SW, respectively [38]. Water was then added to this mixture and stirred into 1-3 cm³ diced mixed rations and dried for preservation.

Chemical reagents and apparatuses

Antibodies purchased from Abcam were: anti-CD95 (ab82419), anti-Fas ligand (ab15285), anti-Bax (ab7977), anti-Bcl-2 (ab7973), anti-caspase-3 (ab32351), anti-caspase-8 (ab25901), anti-caspase-9 (ab32539). Anti-β-actin (BA2305) was purchased from Wuhan Boster Biological Technology, Ltd; peroxidase-conjugated AffiniPure Rabbit anti-goat IgG (H + L) (ZB-2306) was purchased from ZSGB-Bio OriGene. Total ProteoExtract Kit (KGP250), Bradford Protein Assay Reagent Kit (KGA801), and WesternBright Sirius ECL (KGP1125) were all purchased from Nanjing KeyGEN Biotech. CO., LTD. ProteoExtract Cytosol/Mitochondria Fractionation Kit (QIA88) was purchased from Merck. Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore Corp. Equipment utilized include Protein Measuring Instrument (Eppendorf, Germany), High Speed Refrigerated Centrifuge (Sigma, America),

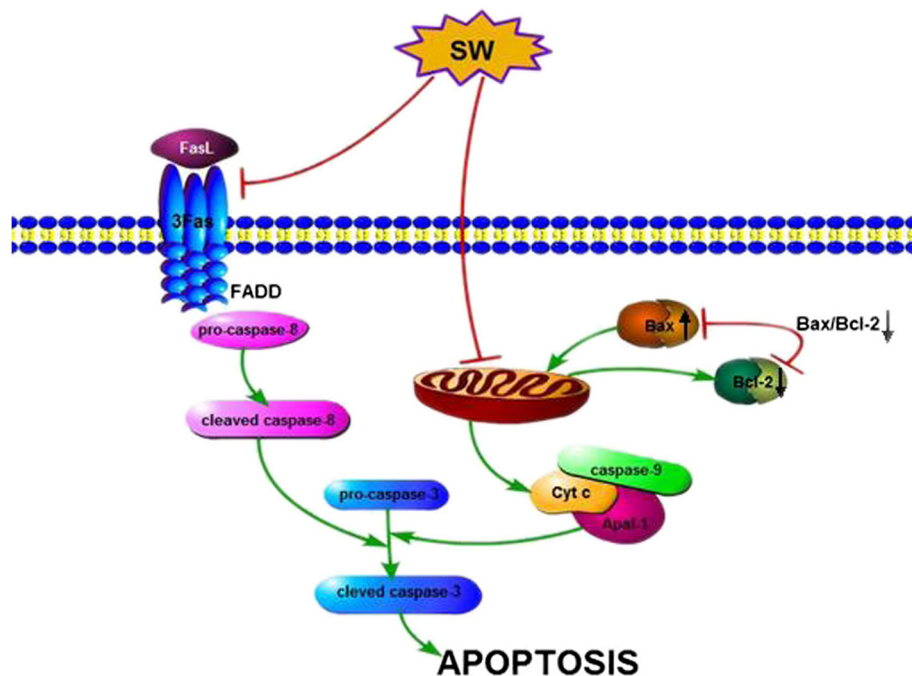


Figure 5 Schematic representation of the apoptotic pathway in *O. kansuensis*-induced SD rat brain cells. Feeding with *O. kansuensis* Bunge induces apoptosis via the activation of Fas/FasL-mediated pathway and the initiator caspase, caspase-8. Bcl-2 was down-regulated and Bax was up-regulated, activating the mitochondria-mediated apoptotic pathway and causing the activation of caspase-9. Finally, the activation of caspase-8 and -9 activates caspase-3, leading to apoptosis.

Instantaneous centrifuge (Kylin-bell) and Micropipette (Eppendorf, Germany).

Establishment of a chronic poisoning model of *O. kansuensis* in SD rats

Experiments were performed in male and female SD rats (200-220 g total body weight). After a week-long adaptation period in a room with controlled temperature ($21 \pm 1^\circ\text{C}$) and lighting (12 h light/12 h dark), 24 SD rats were assigned to either a control group (complete feed) or experimental groups I (15% *O. kansuensis* containing 0.03‰ SW), II (30% *O. kansuensis* containing 0.06‰ SW), or III (45% *O. kansuensis* containing 0.09‰ SW), and 6 SD rats of each group were fed in 3 cages separately. One hundred and nineteen days after poisoning, and all rats showed neurological disorders at different degrees, which were considered to be successfully established a chronic poisoning model of *O. kansuensis*. All rats were anesthetized with ether vapor and sacrificed by decapitation. The blood of all rats were collected and analyzed on concentration of SW in serum by HPLC (The data were not shown). The cerebrum from each animal was collected and preserved in liquid nitrogen.

Western blot analysis

Total protein was collected by Total ProteoExtract Kit (KGP250). Isolation and extraction of mitochondria/cytosol protein was performed using the ProteoExtract Cytosol/Mitochondria Fractionation Kit (QIA88). Protein concentrations were measured using the Bradford Protein Assay Reagent Kit (KGA801). Equivalent amounts of protein were loaded separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 120 V for 90 min. Proteins were subsequently transferred to PVDF membranes at 200 mA for 45 min. PVDF membranes were first blocked with 5% nonfat dry milk at room temperature for 2 h, then incubated with primary antibodies overnight at 4°C , and finally probed by HRP-conjugated secondary antibodies at room temperature for 2 h. The signal was detected using ECL reagent. Quantification was performed by Image system (Bio-Rad) from three independent experiments and analyzed with Quantity One (Bio-Rad).

Statistical analysis

Results are expressed as mean \pm standard deviation. All data were analyzed in SPSS 18.0 using one-way analysis of variance (ANOVA) followed by Duncan's test for multiple comparisons. $P < 0.05$ was considered significant.

Abbreviations

SD: Sprague-Dawley; SW: Swainsonine; PVDF: Polyvinylidene difluoride; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; FADD: Fas-associated death domain; HRP: Horseradish peroxidase; ECL: Electrochemiluminescence; TNF α : Tumor necrosis factor alpha

receptor; DR3: Death receptor 3; DR4: Death receptor 4; DR5: Death receptor 5; AIF: Apoptosis inducing factor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LH conceived the study, carried out the animal experiment, participated in western blot analysis and the statistical analysis and drafted the manuscript. ZL participated in western blot analysis and helped to draft the manuscript. WSS and WWL participated in the animal experiment. ZBY participated in the design of the study and supervised the animal experiment. All authors read and approved the final manuscript.

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