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Swainsonine-induced apoptosis pathway in cerebral cortical neurons

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ABSTRACT

Swainsonine (SW) is an indolizidine alkaloid, and the principal toxic component of the poisonous legume plants *Astragalus* and *Oxytropis* sp. Animals that consume the toxic plants show neurologic symptoms. In this study, the cerebral cortical neurons of primary culture were treated for 12 h with various concentrations of SW. The $[Ca^{2+}]_i$ and the protein expression of caspase-3, -8, -9 and -12 were assessed in all experimental groups. In comparison with the control group, $[Ca^{2+}]_i$ increased significantly in SW-treated groups ($P < 0.05$). SW significantly increased the expression of activated protein caspase-3, -8 and -12 ($P < 0.05$), while caspase-9 did not change ($P > 0.05$). The results suggest that SW induced the apoptosis of neurons through a death receptor pathway and endoplasmic reticulum stress.

Locoweeds are toxic species of the genera *Astragalus* and *Oxytropis* that specifically contain the 1, 2, 8-trihydroxyindolizidine alkaloid, swainsonine (SW) (Dorling et al., 1980; Molyneux and James, 1982). SW causes characteristic clinical symptoms of loco-disease of grazing animals (James and Panter, 1989) associated with locoweed poisoning (Liu, 2006) which includes a slow staggering gait, a rough hair coat, a staring gaze, emaciation, lack of muscular coordination, and extreme nervousness.

Our previous *in vivo* research suggested that SW induces apoptosis in brain cells through death receptor and mitochondria-mediated, caspase-dependent apoptotic pathways in the brain tissue of SD rats (Lu et al., 2013). However, the neurotoxic mechanisms *in vitro* of SW are still unclear. Pang et al. (2012) developed a primary culture of cortical neurons that serves as a model system for SW toxicity, in which treated cell bodies shrink and axons shorten. In addition, Sun et al. (2009) reported that SW induces the apoptosis of glioma cells. We speculated that the neurotoxicity mechanism *in vitro* of SW might be connected with apoptosis according to our previous studies (Pang et al., 2012). Therefore, we conducted a series of experiments to determine the $[Ca^{2+}]_i$ and the expression of apoptosis-regulated proteins. The aim of the work was to understand the mechanism of SW-induced apoptosis of neurons in SD rats *in vitro*, and SW-induced neurotoxic effects.

SW was isolated from *Oxytropis kansuensis* Bunge (a locoweed widely distributed in western China) that was identified by interpretation of spectral data (MS, ^1H NMR, ^{13}C NMR, 2D NMR) as described previously (Tong et al., 2007, 2008). Its purity was 98.17%. The alkaloid was dissolved in Ca^{2+}- and Mg^{2+}-free PBS (0.01 M, pH 7.2) to produce a 10 mg/mL stock solution that was sterilized by ultrafiltration and stored at −20 °C. An animal protocol for ethical use of animals for research was approved by the Laboratory Animals Center of Xi'an Jiaotong University (License No. SCXK (Shaan) 2007-001). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the State Scientific and Technological Commission of China (File No. 2, 1988). Cerebral cortical neurons were prepared as previously described (Silva et al., 2006) from newborn SD rats in DMEM/F_{12} medium containing 10% FBS, and 1% penicillin and streptomycin. On the 3rd day of culture, 2.5 μg/mL Ara-C was added for 24 h to prevent glial and fibroblast proliferation. The neurons were subsequently maintained in serum-free neurobasal medium containing 2% B-27 supplement, and 1% glutamine and 1% penicillin and streptomycin at 37 °C in a humidified incubator with 5% CO_2. The neurons were given fresh media every 2–3 days.

After the neurons were incubated for 4 days, SW of 0, 0.5, 1.0, 2.0 mg/mL was added to the neurons for 12 h. Fluo-3 (10 μmol/L), AM working solution was added to the neurons and incubated for 30 min at 37 °C. The Fluo-3, AM working solution was subsequently discarded and the neurons were washed 3 times with cold PBS. The slides covered with neurons were used for scanning images and quantitative analysis under the Laser Scanning Confocal Microscope. The total proteins from the neurons of each experimental group were collected using a Total ProteoExtract Kit (KGP250). Protein concentrations were measured using a Bradford Protein Assay Reagent Kit (KGA801). Equivalent amounts of proteins were run on a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) at 120 V for 90 min. Subsequently, proteins were transferred to PVDF membranes, at 200 mA for 45 min. After blocking with 5% nonfat dry milk at room temperature for 2 h,

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membranes were incubated with rabbit polyclonal anti-β-actin, rabbit polyclonal anti-caspase-3, -8, -9, -12 antibodies (1:1,000; Abcam) overnight at 4 °C, and then reacted with anti-rabbit secondary antibodies, HRP-conjugated IgG (1:2,000; Zhongshan, Beijing) at room temperature for 2 h. Immunoreactivity was detected using luminol reagent. All data were analyzed in SPASS 18.0 using a one-way analysis of variance (ANOVA) followed by Duncan's test for multiple comparisons. Means with different superscripts differ significantly ($P < 0.05$).

Compared with the control group, $[Ca^{2+}]_i$ increased dramatically in the SW treatment groups ($P < 0.05$). The most obvious increase was with 2 mg/mL SW, referred to the control group and the 0.5 mg/mL group ($P < 0.05$) (Fig. 1). Compared with the control group, the expressions of cleaved-caspase-8 and -12 increased in each treatment group ($P < 0.05$) (Fig. 2). The activated caspase-9 was not detected after treating the cortical neurons with different concentrations of SW for 12 h, and the expression of pro-caspase-9 did not distinctly change ($P > 0.05$) (Fig. 2). The expression of cleaved-caspase-3 protein increased in each treatment group. This expression in the 0.5 mg/mL and 2.0 mg/mL SW group was higher than in the control group ($P < 0.05$), which was higher in the group treated with 1.0 mg/mL SW than the control group, however, the difference was not significant ($P > 0.05$) (Fig. 2).

Early studies demonstrated that SW could induce the apoptosis of human lung cancer cell A549, gastric carcinoma cell SGC-7901 and glioma cell C6 (Li et al., 2012; Sun et al., 2007, 2009). Signal transduction pathways of apoptosis include the death receptor pathway and mitochondrial pathway, both of which can be activated by cascade reactions of caspases and then mediate apoptosis. In the death receptor pathway, death receptors combine with extracellular cognate ligands and the cytoplasmic domain of death receptors (death domain), by collecting adapter protein FADD. In this process, several caspase-8 precursors are collected to form DISC. In different kinds of cells, ligands, combined with death receptors, can amass large amounts of caspase-8. By cutting

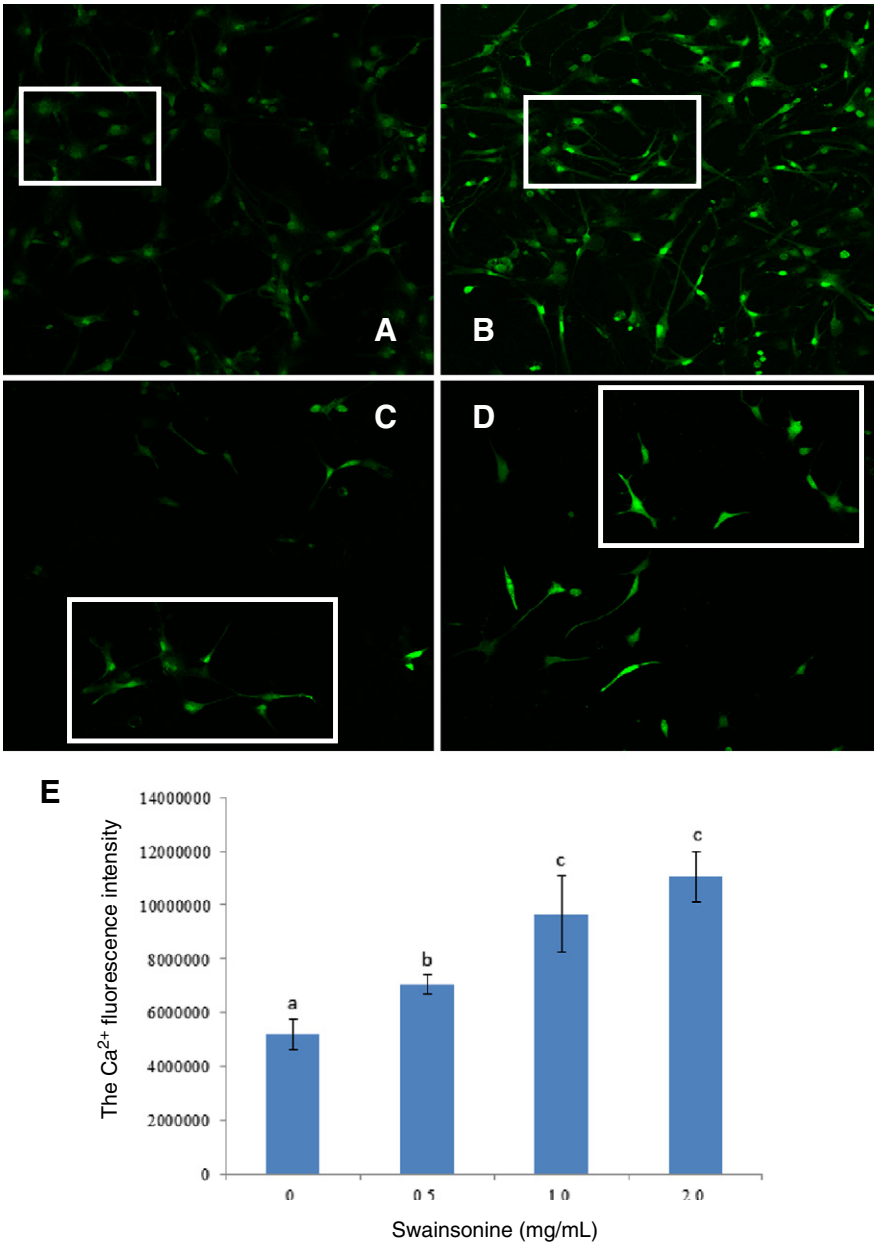

Fig. 1. Effects of swainsonine treatment on $[Ca^{2+}]_i$ of cerebral cortical neurons in 12 h. A: treated with 0 mg/mL SW; B: treated with 0.5 mg/mL SW; C: treated with 1 mg/mL SW; D: treated with 2 mg/mL SW; E: the result of statistical analysis on $[Ca^{2+}]_i$. Values are shown as means ± SEM. The data shown are representative of three independent experiments. Different letters indicate significant difference ($P < 0.05$), while the same letters were not significantly different ($P > 0.05$).