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Pine pollen inhibits cell apoptosis-related protein expression in the cerebral cortex of mice with arsenic poisoning★

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Abstract

Previous studies have demonstrated that pine pollen can inhibit cerebral cortical cell apoptosis in mice with arsenic poisoning. The present study sought to detect the influence of pine pollen on apoptosis-related proteins. Immunohistochemistry, western blotting and enzyme-linked immunosorbent assays were used to measure the levels of apoptosis-related proteins in the cerebral cortex of mice with arsenic poisoning. Results indicated that pine pollen suppressed cell apoptosis in the cerebral cortex of arsenic-poisoned mice by reducing Bax, Bcl-2 protein expression and increasing p53 protein expression.

Key Words: pine pollen; arsenic poisoning; apoptosis; cerebral cortex; neural regeneration

Abbreviations: ELISA, enzyme-linked immunosorbent assay; MAPK, mitogen-activated protein kinase

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INTRODUCTION

The mechanism of arsenic poisoning has attracted growing interest[1-3]. Pine pollen is effective in treating hyperplasia of the prostate gland, protecting the liver, reducing blood fat, improving immunity and relieving fatigue[4-5]. A previous study reported that pine pollen can reduce renal cell apoptosis in arsenic poisoned mice[6]. Studies focusing on arsenic-induced liver and renal cell apoptosis concluded that Bcl-2 is an anti-apoptotic factor[7], and morphological studies have been used to determine damage to the brain by arsenic[8-9]. In addition, the apoptosis-related factor, Fas and Bcl-2 are linked to immunological tolerance[9].

Pine pollen extract has significant anti-tumor effects *in vivo* and *in vitro* [10-11]. Inorganic arsenic can influence nerve reactions in mice[12], and can induce hippocampal nerve cell apoptosis by upregulating Bax and downregulating Bcl-2 proteins[13]. In addition, arsenate can selectively activate p38/mitogen-activated protein kinase 14 (MAPK14) and c-Jun N-terminal kinase 3, inducing cell apoptosis in the mouse cerebellum[14].

The present study utilized immunohistochemistry and western blotting techniques to detect cell apoptosis and determine expression of apoptosis-related factors, Bax, Fas, Bcl-2, p38 and p53 in the cerebral cortex of mice treated with pine pollen after arsenic poisoning.

RESULTS

Quantitative analysis of experimental animals

A total of 54 adult mice were randomly assigned to normal control, treatment and $NaAsO_2$ control groups, with 18 animals in each group. The normal control group was treated with distilled water, whereas treatment and $NaAsO_2$ control groups were injected with $NaAsO_2$ every 2 days to establish arsenic poisoning. The treatment group was intragastrically perfused with pine pollen from day 15 after the initiation of arsenic poisoning until 45 days after the beginning of arsenic poisoning[6]. All mice were analyzed at 45 days after initiation of arsenic poisoning. All 54 mice were included in the final analysis.

Pine pollen suppressed cell apoptosis in the cerebral cortex of mice with arsenic poisoning

Flow cytometry showed the percentage of apoptotic cells (6.95 ± 0.01%) in the treatment group was significantly lower than in the $NaAsO_2$ control group (21.64 ± 0.50%, $P < 0.05$), but similar to that of the normal control group (2.49 ± 0.02%; $P > 0.05$) at 30 days following pine pollen treatment.

Influence of pine pollen on apoptosis-related factors Bax, Fas and Bcl-2 levels in the cerebral cortex of mice with arsenic poisoning

Enzyme-linked immunosorbent assay (ELISA) showed less Bax and Bcl-2 content in the treatment group compared with the $NaAsO_2$ control group at 30 days following pine pollen treatment ($P < 0.05$; Table 1). Levels of Fas were slightly lower in the pollen treated group compared with the $NaAsO_2$ control groups (Table 1).

Influence of pine pollen on p38 protein expression in the cerebral cortex of mice with arsenic poisoning

p38 is a proapoptotic factor. p38 expression in cells of the cerebral cortex was similar between the treatment and $NaAsO_2$ control groups at 30 days following pine pollen treatment (absorbance ratio to β-actin: 1.152 ± 0.050 *vs*. 1.644 ± 0.440), and slightly greater than the normal control group (0.423 ± 0.205; Figure 1).

p53 protein expression levels in the cerebral cortex of mice with arsenic poisoning

p53 protein is an anti-apoptotic factor. Immunohistochemistry showed that p53 protein expression in the cerebral cortex of mice was present in the control and $NaAsO_2$ control groups but was increased in the pollen treated group at 30 days following pine pollen treatment (Figure 2).

Table 1 Influence of pine pollen treatment on Bax, Fas and Bcl-2 levels in the cerebral cortex of arsenic-poisoned mice

Group	Bax (pg/mL)	Fas (μg/mL)	Bcl-2 (pg/mL)
Normal control	3 070.3±509.2[a]	3.31±0.26[a]	3 477.0±1620.8[a]
Treatment	3 259.0±407.1[a]	4.66±0.45	1 838.6±406.8[a]
$NaAsO_2$ control	3 945.1±181.5	6.25±0.56	7 121.2±3 492.6

[a]$P < 0.05$, *vs*. $NaAsO_2$ control group (two-sample *t*-test). Data were expressed as mean ± SD of 18 mice in each group.

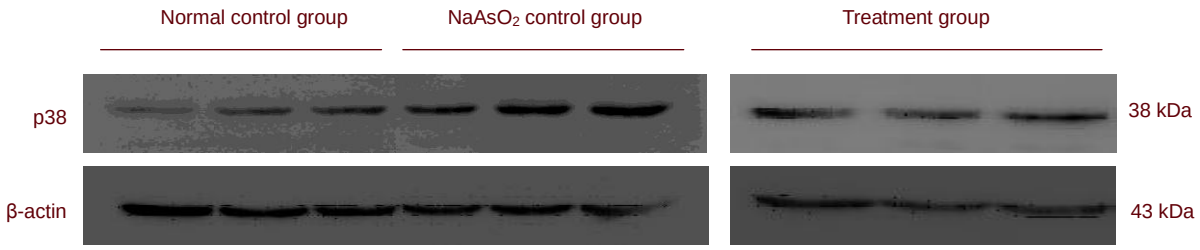

Figure 1 p38 protein expression in cells of cerebral cortex in mice. The experiment was performed in triplicate.

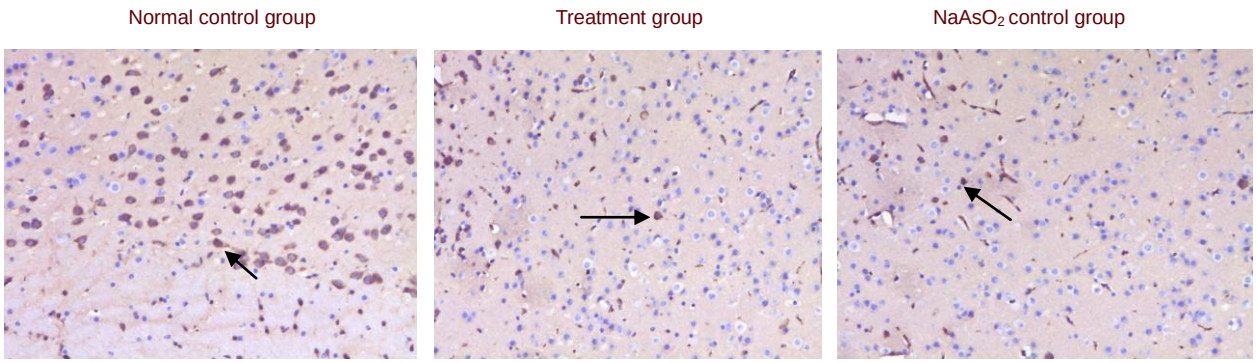

Figure 2 p53 protein expression in the cerebral cortex of mice (immunohistochemical staining, × 400). Positive p53 expression is represented as a brown yellow stain (arrows). High levels of p53-positive cells were observed in the normal and $NaAsO_2$ control mice. There were increased numbers of p53-positive cells in the treatment group compared with $NaAsO_2$ control group.

DISCUSSION

In the present study, flow cytometry showed that pine pollen reduced the cell apoptosis rate in the cerebral cortex of mice with arsenic poisoning. In addition, we detected the expression of apoptosis-related factors, Bax, Fas, Bcl-2 and p53. Arsenic has been shown to induce nerve cell apoptosis in the hippocampus, and Bcl-2 is an anti-apoptotic factor[13]. However, results from the present study suggested that cortical Bcl-2 and Fas content was higher in the $NaAsO_2$ control group compared with normal control and treatment groups, indicating Bcl-2 may be an apoptosis-inducing factor. Bcl-2 may also be associated with the Fas pathway[15-17] during cell apoptosis in the cerebral cortex of mice with arsenic poisoning. However, the precise mechanism of how these pathways interact remains unknown.

In conclusion, treatment with pine pollen can downregulate cortical Bax and Bcl-2 expression, suggesting that pine pollen may be a useful treatment for arsenic poisoning. However, levels of p53, a pro-apoptotic protein, were also increased, thus further studies are needed to confirm the precise mechanism of pine pollen treatment.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

The experiment was performed at the Central Laboratory, Youjiang Medical College for Nationalities, China, in October 2011.

Materials

Animals

A total of 24 healthy, female Kunming mice, aged 12 weeks, weighing 23–25 g, were purchased from the Department of Laboratory Animal, Guangxi Medical University (license No. SCXK (Gui) 2009-0002). Experimental procedures were performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, issued by the Ministry of Science and Technology of China[18].

Drugs

Thirty packages × 3 g pine pollen disruption powder (Xinshidai Company, Yantai, Shandong, China; G20070244), were used to prepared 100% pine pollen powder following disruption at low temperature, with a disruption rate of 100%. Pine pollen was administrated to mice by intragastric perfusion (by placing the perfusion device through the mouth and directly into the stomach). Pine pollen is composed of over 200 nutrition elements, including 22 amino acids, 15 vitamins, 30 minerals, 100 enzymes, nucleic acids, unsaturated fatty acids, lecithin, flavonoids, monosaccharides and polysaccharides. $NaAsO_2$ and analytical reagents were purchased from the Fourth Branch of Shanghai Chemical Reagent Company, China.

Methods

Establishment of arsenic poisoning model

The treatment and $NaAsO_2$ control groups were injected with $NaAsO_2$ (1 mg/kg), every 2 days to induce arsenic poisoning. Blood arsenic content in each group was determined 15 days after arsenic poisoning induction. Successful poisoning was represented by a significant difference in the blood arsenic content between normal and arsenic poisoning induction groups. In addition, the treatment group was intragastrically perfused with pine pollen, 1 g/kg, for 30 days.

Flow cytometry for cell apoptosis in the cerebral cortex of mice

Following pine pollen treatment for 30 days, the mice were anesthetized and sacrificed. The cerebral cortex was harvested, dispersed by pipetting, filtrated, centrifuged, rinsed, and adjusted to density of 1×10^6 cells/L. Cells were treated according to a cell apoptosis kit (Boster, Wuhan, China), and the apoptosis rate was determined using BD FACScant II automatic flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

ELISA for Bax, Fas and Bcl-2 levels in cell supernatant of the cerebral cortex

The cerebral cortex was harvested, centrifuged, mixed with protein extraction cell lysate, suspended and centrifuged to extract protein samples. Mouse apoptosis factors Bax and Fas ELISA kit (Invitrogen Life Technologies, Carlsbad, CA, USA) and ELISA Bcl-2 kit (Shanghai Westang Bio-Tech Inc., Ltd., Shanghai, China) were used according to the manufacturer's instructions. Cells were incubated with primary antibodies, mouse anti-Bax, Fas, Bcl-2 monoclonal antibody, at 37°C for 1 hour, followed by secondary antibodies, biotin labeled goat anti-mouse Fas polyclonal antibody at 37°C for 30 minutes. Absorbance at 450 nm was determined using RT-60000 microplate reader (Rayto Life and Analytical Sciences Co., Ltd., Guangdong, China). A standard curve was drawn according to the absorbance values and corresponding concentration of standard samples, and a linear equation was obtained. Sample concentrations were calculated with the linear equation using the absorbance value of each group sample.

Western blot for p38 protein expression in cortical cells of mice

p38 protein expression was detected by western blot assay (Daan Gene Co., Ltd., Guangzhou, China). Briefly, cerebral cortex tissues, 0.3 g, were lysed using radioimmunoprecipitation assay buffer and cut into pieces, followed by 22% amplitude ultrasound. The tissue solution was then rotated and extracted in the refrigerator for 30 minutes, and centrifuged at 16 000 r/min, 4°C for 30 minutes. The supernatant was harvested, electrophoresed and stained with Coomassie brilliant blue to quantify sample amount. The samples were stored at −80°C, followed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein

was transferred to polyvinylidene fluoride membrane (Beijing Neuron Biotechnology, Beijing, China) at 350 mA for 1 hour. The membrane was blocked in Tris-buffered saline Tween-20 with 5% non-fat milk powder for 2 hours, washed with Tris-buffered saline Tween-20 three times, for 5 minutes each. The membrane was incubated with primary antibody, rabbit anti-MAPK14 (p38MAPK) polyclonal antibodies (1:1 000; Beijing Biosynthesis Biotechnology, Beijing, China) overnight at 4°C, washed with Tris-buffered saline Tween-20 three times, for 5 minutes each, followed by incubation with secondary antibody horseradish peroxidase-labeled goat anti-mouse antibody (1:5 000; Shanghai Yiboju, Shanghai, China) at room temperature for 1 hour. The membrane was washed with Tris-buffered saline Tween-20, incubated with chemiluminescence reagent A and B solution at equal volume for 1 minute, and imaged using gel imaging system (Bio-Rad, Hercules, CA, USA). Absorbance ratio of samples to internal reference (Mouse Anti-β-actin antibody, Boster) was calculated.

Immunohistochemistry for p53 protein expression in the cerebral cortex

Cerebral cortex paraffin sections were prepared, dewaxed, hydrated, washed with phosphate buffered saline (PBS), treated with high pressure to retrieve antigen, washed with PBS, incubated with primary antibody rabbit anti-p53 polyclonal antibody (200 μg/mL) overnight at 4°C, followed by incubation with secondary antibody, horseradish peroxidase-labeled anti-rabbit antibody (10 μg/mL), at 30°C for 30 minutes. The sections were washed with PBS, stained, washed with PBS, dehydrated, cleared and mounted. Positive p53 staining was represented by brown yellow color of sections, and observed by light microscopy (Olympus, Tokyo, USA).

Statistical analysis

Data were analyzed using SPSS version 13.0 (SPSS, Chicago, IL, USA) and was expressed as mean ± SD. Intragroup mean differences were compared utilizing two-sample *t*-test. A value of $P < 0.05$ was considered statistically significant.

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Author contributions: Yanhong Luo conceived and designed this study, conducted experiments, and wrote manuscript. Yaodong Wei revised the manuscript. Taizhong Wang performed statistical analyses. Dongzhu Chen, Tiansheng Lu, Ruibo Wu, and Keke Si established the animal model.

Conflicts of interest: None declared.

Ethical approval: This study received permission from the Animal Ethnics Committee of Youjiang Medical College for Nationalities, China.

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