

Original Article

# Growth inhibitory effects and molecular mechanisms of crotoxin treatment in esophageal Eca-109 cells and transplanted tumors in nude mice

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**Aim:** To investigate the antitumor actions of the *Crotalus durissus* neurotoxin (crotoxin) on human esophageal carcinoma (Eca-109) cells *in vitro* and transplanted esophageal Eca-109 tumors in nude mice.

**Methods:** The growth-inhibitory effect was analyzed in Eca-109 cells using MTT assay. Cell morphology changes in nuclei were observed using Hoechst 33342 staining, while apoptosis and cell cycle distribution were examined by flow cytometry. RT-PCR was used to measure the Bcl-2, p15, and caspase-3 p17 gene expression levels. A tumor transplantation model was established by inoculation of Eca-109 cells into female Balb/c nude mice. Crotoxin (25, 50, and 100 mg/kg) was subcutaneously injected into the transplanted tumors every 2 d for a total of 10 injections. Tumor size and weight were measured. Bcl-2, p15, and caspase-3 p17 protein expression in transplanted tumors was analyzed using Western blotting.

**Results:** Crotoxin (25, 50, and 100 µg/mL) inhibited the growth of Eca-109 cells in a dose-dependent manner with inhibition rates of 22.9%, 35.8%, and 57.2%, respectively. Hoechst 33342 staining revealed apoptotic cells with pyknotic nuclear chromatin after crotoxin treatment. In Eca-109 cells, crotoxin induced apoptosis and G<sub>1</sub> block, significantly upregulated the expression of p15 and caspase-3 p17 genes and downregulated the expression of Bcl-2 gene. Furthermore, crotoxin inhibited the growth of Eca-109 tumors in nude mice in a dose-dependent manner. Western blotting showed that crotoxin increased p15 and caspase-3 p17 protein levels and reduced Bcl-2 protein level in tumor specimens.

**Conclusion:** Crotoxin inhibits the growth of Eca-109 cells *in vitro* via apoptosis induction and G<sub>1</sub> block. Local administration of crotoxin inhibits the growth of subcutaneously transplanted Eca-109 cells in nude mice, possibly via increasing p15 and caspase-3 p17 protein expression and reducing Bcl-2 protein expression.

**Keywords:** *Crotalus durissus* neurotoxin (Crotoxin); human esophageal Eca-109 cells; apoptosis; p15; caspase-3 p17; Bcl-2; G<sub>1</sub> phase block; transplant tumor

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## Introduction

Esophageal tumors are one of the most common malignant tumors. Treatment usually involves surgery, but the cure rate remains unsatisfactory. Therefore, much attention has been focused on the development of drug treatments for esophageal cancer, leading to important advances, including the identification of components of natural substances with antitumor activities<sup>[1–5]</sup>. *Crotalus durissus* neurotoxin (crotoxin) is a nerve toxin isolated from South American diamondback snake

subfamilies. Recent studies have indicated a potential anti-tumor effect of crotoxin<sup>[6,7]</sup>, although its specific mechanism remains unclear. In this study, we therefore investigated the antitumor effects of crotoxin in human esophageal carcinoma (Eca-109) cells and in a nude mouse tumor transplantation model. We also discuss the possible molecular mechanisms responsible for crotoxin's observed actions in order to provide a theoretical basis for future clinical applications.

## Materials and methods

### Cell culture

Human esophageal carcinoma (Eca-109) cells were purchased from the Shanghai Institutes for Biological Sciences and cultured in RPMI-1640 medium containing 10% fetal bovine

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serum at 37°C in a 5% CO<sub>2</sub> incubator. Cells were grown as an adherent monolayer and were digested with 0.25% trypsin and passaged when they reached 80%–90% confluence. Experiments were conducted using cells in the logarithmic phase of growth.

#### Drugs, reagents, and instruments

Crotoxin was a gift from the Pharmacy Department of Suzhou University Medical College (Suzhou, China). Dimethyl sulfoxide (DMSO) and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma (St Louis, MO, USA). The flow cytometer was from BD (Franklin Lakes, NJ USA). The microplate reader was from Bio-Rad (Hercules, CA, USA).

#### Cell-growth inhibition experiments (MTT)

Cells were harvested during logarithmic growth and digested with 0.25% trypsin to yield a single-cell suspension containing  $1.0 \times 10^5$  cells/mL. Cells were then inoculated into 96-well plates at a density of 100  $\mu$ L/well. Six parallel wells were used for each experiment. Cells were allowed to adhere for 24 h, after which the culture medium was replaced with medium containing 25, 50, or 100  $\mu$ g/mL crotoxin (experimental groups); 5, 10, or 20  $\mu$ g/mL cisplatin (positive control groups); medium alone (negative control group); or nothing (blanks). After 24 h, MTT (5 mg/mL, 20  $\mu$ L) was added to each well followed by incubation for another 4 h. DMSO (150  $\mu$ L) was then added to each well, and the plates were vibrated gently for 10 min. Absorption at 570 nm (*OD*) was measured using a microplate reader. Each experiment was repeated three times, and the average values were calculated. Cell proliferation inhibition (%) =  $(1 - \text{experimental group average } OD \text{ value} / \text{control group average } OD \text{ value}) \times 100\%$ .

#### Hoechst 33342 staining of Eca-109 cells

Adherent Eca-109 cells in the logarithmic growth phase were digested with 0.25% trypsin to produce a single-cell suspension. The cell density was adjusted to  $1.0 \times 10^5$  cells/mL, and a total of  $5 \times 10^4$  cells were inoculated into each well of a 24-well plate and cultured overnight in RPMI-1640 containing 10% fetal calf serum. After the cells had adhered, the original medium was removed and replaced with medium containing 25, 50, or 100  $\mu$ g/mL crotoxin in 1 mL complete RPMI-1640 medium (a total of 9 wells) and with complete RPMI-1640 without crotoxin in three negative-control wells. The cells were cultured for an additional 24 h and adjusted to pH 7.0, and 1 mg/mL Hoechst 33342 was added to a final concentration of 20  $\mu$ g/mL. Cells were incubated at 37°C for 30 min, followed by the addition of 4% polyphosphate formaldehyde mixed with culture medium in a 3:1 ratio, yielding a final formaldehyde concentration of 1%. Cells were fixed in this solution for 5–10 min, and the wells were then examined and photographed under a fluorescence microscope.

#### Detection of effects of crotoxin on cell cycle by flow cytometry

Eca-109 cells were treated in one of five ways: negative control group (no drug intervention), crotoxin group 1 (25  $\mu$ g/mL),

crotoxin group 2 (50  $\mu$ g/mL), crotoxin group 3 (100  $\mu$ g/mL), and cisplatin group (10  $\mu$ g/mL). Eca-109 cells from each group were digested with 0.25% of trypsin and centrifuged at 1500 r/min for 5 min to produce a clear supernatant. A total of 0.5 mL of sediment was oscillated to separate the cells, and the cells were then fixed in 70% ethanol at -20°C for 24 h. Before each experiment, the fixed cells were centrifuged at 1500 r/min for 10 min and washed twice in phosphate-buffered saline. A total of 200  $\mu$ L RNaseA (1 g/L) was added, and the samples were digested at 37°C in a constant-temperature water bath for 30 min and immediately immersed in ice water to terminate RNase activity. One milliliter of propidium iodide (50  $\mu$ g/mL) was added, and the samples were incubated at 4°C for 30 min while protected from light. The cell cycle distribution was examined using a flow cytometer.

#### Analysis of Bcl-2, p15, and caspase-3 p17 gene expression levels by real-time quantitative fluorescence transcriptase polymerase chain reaction (RT-PCR)

Eca-109 cells in each experimental group were cultured with crotoxin at final concentrations of 25, 50, and 100  $\mu$ g/mL, while negative control cells were cultured in RPMI-1640 without crotoxin for 48 h. Total RNA was extracted according to standard RT-PCR protocol and resolved by 2.5% agarose gel electrophoresis. p15, Bcl-2, and caspase-3 p17 DNA was amplified using the corresponding primers: 5'-TTA GCA CTT GGG TGA CGG-3' and 5'-TCG CTT GCA CAT CCT CTC-3' for p15, 5'-GGG AGA ACA GGG TAC GAT AA-3' and 5'-CCA CCG AAC TCA AAG AAG G-3' for Bcl-2, 5'-GTG CGA GTG TCT AAC GG-3' and 5'-CGG ATC AGT CTT TGG GTC-3' for caspase-3 p17, and 5'-GTG GAC ATC CGC AAA GAC-3' and 5'-GAA AGG GTG TAA CGC AAC T-3' for  $\beta$ -actin.

#### Nude mouse tumor transplantation model

Eca-109 cells undergoing logarithmic growth were adjusted to a cell density of  $1.0 \times 10^8$  cells/mL. Twenty-eight 4–6 weeks old female Balb/c nude mice (weight 16–20 g, average weight 17.5 g) were used. Anesthesia was induced by intraperitoneal injection of 2 mg/kg lidocaine. The shoulder skin was disinfected and each animal received a subcutaneous injection of a 125- $\mu$ L cell suspension containing  $1.25 \times 10^7$  cells. After inoculation, animals were maintained in specific pathogen-free conditions, and their activity, mental state, feeding and defecation were observed daily. Tumor size was measured using Vernier calipers, and the mice were weighed twice per week.

#### Calculation of tumor inhibition by crotoxin

All experimental animals developed nodules of approximately 4–6 mm in diameter within 15 d of inoculation. The animals were then divided randomly into a high-dose group (100 mg/kg crotoxin), a medium-dose group (50 mg/kg crotoxin), a low-dose group (25 mg/kg crotoxin) and 4 negative control groups (which received equivalent doses of 0.9% NaCl). Each group contained 7 mice. The experimental groups received subcutaneous injections of crotoxin into the transplanted tumors every 2 d for a total of 10 injections, while the nega-

tive control mice received subcutaneous injections of the same volume of saline. Mice were euthanized after 4 weeks, blood samples were taken, and the subcutaneous tumors were removed and weighed. The tumor inhibition rates were calculated as follows: tumor inhibition rate (%)=(1-weight of experimental group tumor/weight of control group tumor)×100%. Tumor tissues were processed for immunohistochemistry using conventional methods.

#### Western blot analysis of Bcl-2, p15, and caspase-3 p17 protein expression in transplanted tumors

Approximately 100 mg of transplanted tumor tissue was removed from liquid nitrogen, quickly placed into 2 mL lysate and fully homogenized. The mixture was transferred to an EP tube on ice and subjected to ultrasonic irradiation for 30 min, followed by centrifugation at 12000 r/min for 5 min at 4°C. The concentration of proteins in the supernatant was determined using the BCA method. The samples were boiled, and the proteins were separated by SDS-PAGE. Blots were transferred to nitrocellulose membranes and hybridized. The primary antibodies used were mouse anti-human p15 monoclonal antibody, mouse anti-human caspase 3-p17 monoclonal antibody, and mouse anti-human Bcl-2 monoclonal antibody, with β-actin as an internal control. Secondary antibodies were labeled with horseradish peroxidase. Protein expression was analyzed by chemiluminescence.

#### Statistical analysis

Measurements are expressed as the mean±SD. Differences between experimental and control groups were compared using two-sample mean difference *t*-tests with SPSS (Statistical Package for the Social Sciences) 15.0 statistical software for single-factor analysis of variance. *P*<0.05 was considered to represent a significant difference.

## Results

### Effects of crotoxin on growth of esophageal carcinoma Eca-109 cells

All concentrations of crotoxin significantly inhibited the growth of Eca-109 cells compared to the control group (*P*<0.05). The growth-inhibitory activity of crotoxin was dose-dependent with inhibition rates of 22.9%, 35.8%, and 57.2%, respectively (Table 1). Cisplatin also inhibited the growth of Eca-109 cells compared to the control group (*P*<0.05), with inhibition rates of 12.0%, 18.3%, and 32.4%, respectively (Table 2).

**Table 1.** Effects of crotoxin on the growth of Eca-109 cells. Mean±SD. *n*=3. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs control.

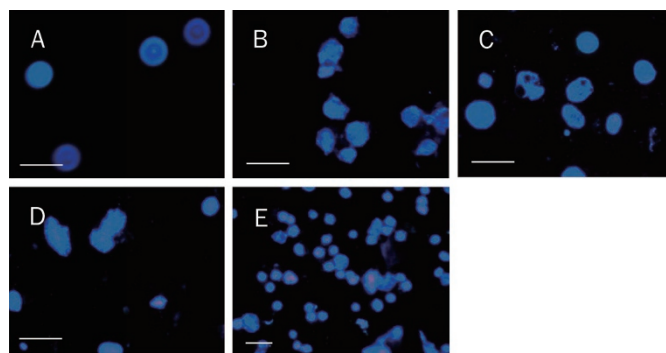
Concentration of crotoxin (μg/mL)	OD value	<i>P</i> value	Inhibition rate
Control group	0.732±0.091		
25	0.574±0.115 <sup>b</sup>	0.033	22.9%
50	0.492±0.065 <sup>c</sup>	0.002	35.8%
100	0.364±0.055 <sup>c</sup>	0.001	57.2%

**Table 2.** Effects of cisplatin on the growth of Eca-109 cells. Mean±SD. *n*=3. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs control.

Concentration of cisplatin (μg/mL)	OD value	<i>P</i> value	Inhibition rate
Control group	0.865±0.077		
5	0.767±0.060 <sup>b</sup>	0.047	12.0%
10	0.601±0.062 <sup>b</sup>	0.018	18.3%
20	0.569±0.089 <sup>c</sup>	0.003	32.4%

### Effects of crotoxin on Eca-109 cell apoptosis using Hoechst 33342 staining

In normal Eca-109 cells, nuclei demonstrated diffused and even fluorescence. However, after crotoxin treatment, cell nuclei became pyknotic and agglomerated, some cells burst, and nuclear debris was scattered between the cells. Large, dense, hyperchromatic, fluorescent particles or pieces were observed in the nuclei or cytoplasm of apoptotic cells (Figure 1).



**Figure 1.** Effects of crotoxin and cisplatin on Eca-109 cells using Hoechst 33342 staining. (A) Control; (B) Crotoxin (25 μg/mL); (C) Crotoxin (50 μg/mL); (D) Crotoxin (100 μg/mL); (E) Cisplatin (10 μg/mL). Scale bar=50 μm.

### Effect of crotoxin on cell cycle in Eca-109 cells

Exposure of Eca-109 cells to 25 μg/mL of crotoxin for 24 h increased the diploid peak by 7.45%±1.58% compared to the control group (0.71%±0.11%) (*P*<0.05) and increased the quantity of G<sub>1</sub> phase cells by 70.04%±4.52% compared to the control group (54.88%±7.56%) (*P*<0.05). Treatment with 50 μg/mL of crotoxin for 24 h increased the diploid peak by 8.23%±1.47% and increased the quantity of G<sub>1</sub> phase cells by 71.14%±4.86% compared to the control groups (both *P*<0.05 compared to the control group). Treatment with 100 μg/mL of crotoxin increased the diploid peak by 11.23%±1.51% and increased the quantity of G<sub>1</sub> phase cells by 78.19%±4.90% (both *P*<0.01 compared to the control group). Cisplatin increased the diploid peak by 5.59%±0.13% and increased the G<sub>1</sub> phase population by 65.46%±5.47% compared to the negative control group (both *P*<0.05 compared to the control group) (Table 3 and 4).

**Table 3.** Effects of crotoxin on cell apoptosis rate in Eca-109 cells. Mean±SD. *n*=3. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs control.

Group	Diploid peak (%)	<i>P</i> value
Control group	0.71±0.11	
Crotoxin 25 µg/mL	7.45±1.58 <sup>b</sup>	0.028
Crotoxin 50 µg/mL	8.23±1.47 <sup>b</sup>	0.019
Crotoxin 100 µg/mL	11.23±1.51 <sup>c</sup>	0.001
Cisplatin 10 µg/mL	5.59±0.13 <sup>b</sup>	0.033

**Table 4.** Effects of crotoxin on cell cycle in Eca-109 cells. Mean±SD. *n*=3. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs control.

Group	G <sub>1</sub> phase (%)	<i>P</i> value
Control group	54.88±7.56	
Crotoxin 25 µg/mL	70.04±4.52 <sup>b</sup>	0.010
Crotoxin 50 µg/mL	71.14±4.86 <sup>c</sup>	0.000
Crotoxin 100 µg/mL	78.19±4.90 <sup>c</sup>	0.000
Cisplatin 10 µg/mL	65.46±5.47 <sup>b</sup>	0.028

**RT-PCR analysis of the effects of crotoxin on gene expression in Eca-109 cells**

Gene expression levels of p15 and caspase-3 p17 increased in Eca-109 cells after exposure to crotoxin for 24 h in a dose-dependent manner. In contrast, Bcl-2 gene expression levels decreased with increasing concentration of crotoxin (Table 5).

**Tumor-inhibitory effects of crotoxin on transplanted esophageal Eca-109 tumors in nude mice**

All mice developed palpable, subcutaneous round or oval nodules at the injection site 4–6 d after subcutaneous inoculation of human esophageal carcinoma Eca-109 cells. Nodules were 2–3 mm in diameter with clear boundaries, and they could be removed without skin adhesion. Body weight and activity of the transplanted mice were initially unaffected, but the activity levels decreased slightly with increasing tumor size. There were no significant differences in body weight between the experimental and control groups. Tumor diameter was approximately 5–6 mm at 12–16 d after inoculation. The transplanted tumors grew more slowly in animals treated with local crotoxin injections, and their mood remained good. The weight change remained small 20 d after inoculation, with

no signs of angularity and no deaths. Tumors in the control group grew more rapidly, and 30 d after inoculation, the control animals appeared angular with weight loss, sluggishness, and reduced activity, although no deaths occurred.

Mice were euthanized one day after completion of drug administration, and the subcutaneous nodules were removed. Tumors showed local nodular or lobulated growth. Tumor size was decreased in the crotoxin-treated groups, and these tumors appeared harder, with no obvious new blood vessels on the surface, no obvious surrounding tissue adhesion, and no signs of protrusion into the muscle and rib cage, indicating a degenerative state of the tumors. Tumors in the control saline-treated mice were significantly larger than those in the crotoxin-treated group, with a greater quantity of new blood vessels on the surface and in sections, indicating a rich blood supply. Anatomical observation revealed adhesion of the tumors to the surrounding tissues. No distant metastases were detected in any animals. The tumor-inhibition rates were 10.8%, 35.7%, and 91.7% in the low-, medium- and high-crotoxin-dose groups, respectively. The average weights of tumors in the medium- and high-crotoxin-dose groups were significantly reduced (*P*<0.01, Table 6).

**Table 6.** Effects of crotoxin on tumor weight and tumor inhibition in a nude mouse subcutaneous transplantable tumor model. Mean±SD. *n*=7 mice. <sup>c</sup>*P*<0.01 vs control.

Concentration of crotoxin (mg/kg)	Average tumor weight (g)	Tumor-inhibition rates (%)
Control group	0.75±0.14	–
25	0.68±0.15	10.8
50	0.45±0.10 <sup>c</sup>	35.7
100	0.06±0.08 <sup>c</sup>	91.7

Based on single-factor analysis of variance, tumor weights in the high- and medium-dose groups were lower than those of the control group (*P*<0.01), while tumor weight in the low-dose group was also decreased (*P*>0.05). Tumor weights differed significantly between the high-, medium- and low-dose groups (*P*<0.05).

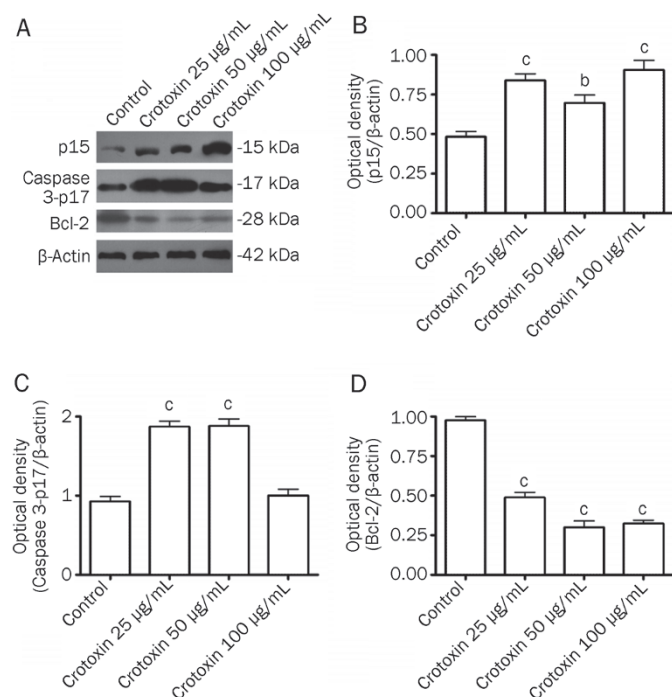
**Effects of crotoxin on p15, caspase-3 p17 and Bcl-2 protein expression**

The protein expression of p15, caspase-3 p17 and Bcl-2 was

**Table 5.** Effects of crotoxin on gene expression in Eca-109 cells.

Concentration of crotoxin	Total number		p15 (+)		Caspase 3 p17 (+)		Bcl-2 (+)	
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
Control group	15	100	1	6.7	1	6.7	11	73.3
Crotoxin 25 µg/mL	15	100	8	53.3	6	40.0	4	26.7
Crotoxin 50 µg/mL	15	100	10	66.7	9	60.0	3	20.0
Crotoxin 100 µg/mL	15	100	13	86.7	12	80.0	2	13.3

determined with immunoblotting. The results showed that crotoxin induced a robust increase in active caspase-3 p17 protein levels and p15 protein levels after treatment with 25 to 50  $\mu\text{g}/\text{mL}$  of crotoxin (Figure 2A–2C). A marked reduction in the Bcl-2 protein level was observed following treatment with 25 to 100  $\mu\text{g}/\text{mL}$  crotoxin (Figure 2A and 2D).



**Figure 2.** Effects of crotoxin on p15, Caspase 3-p17, and Bcl-2 protein expression in Eca-109 cells. Protein levels were analyzed with immunoblotting. Optical densities of respective protein bands were analyzed with Sigma Scan Pro 5 and normalized with loading control ( $\beta$ -actin). Mean $\pm$ SD.  $n=6$ . <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs control group.

## Discussion

The *Crotalus durissus* neurotoxin (crotoxin) is composed of two non-covalently bonded subunits, A and B. Crotoxin B is also known as phospholipase  $A_2$  and is considered to be the main active unit<sup>[8, 9]</sup>, which specifically catalyzes hydrolysis of glycerophosphatide, generating lysophospholipids and fatty acids. Recent studies have reported a possible antitumor function for crotoxin<sup>[10–14]</sup>, although the specific mechanism responsible for this activity remains unclear. However, it is possible that it may act via the release of cytochrome c and the activation of caspase-3, thus destroying the mitochondrial membrane potential of the tumor cells.

The results of this study demonstrated a clear, dose-dependent inhibition of Eca-109 human esophageal carcinoma cell growth by crotoxin. Fluorescence microscopy also revealed increased apoptosis characterized by nuclear pyknosis, apoptotic-body formation and other morphological features, while flow cytometry detected an obvious apoptosis peak and  $G_1$  phase block after treatment of cells with crotoxin for 24 h.

These results suggest that the anti-tumor effect of crotoxin is closely related to apoptosis induction and cell cycle blockade.

The current study employed a range of methods to confirm the effects of crotoxin in promoting apoptosis in Eca-109 cells. Hoechst 33342 staining and fluorescence microscopy demonstrated apoptotic nuclei and hyperchromatic dense particles within the cytoplasm, further confirming the induction of apoptosis-related morphological changes in Eca-109 cells by crotoxin.

Apoptosis is regulated and restricted by many types of mechanisms, including caspase activation. Caspases are homocysteine aspartate-specific proteases, of which caspase-3 is one of the most important executors of apoptosis. Caspase-3 is responsible for cutting all or part of the key proteins during the implementation stage of apoptosis. Once caspase-3 is activated, cell apoptosis is irreversible<sup>[15, 16]</sup>. Previous studies have shown that caspase-3 activation is an irreversible step in apoptosis and that apoptosis is closely related to tumor occurrence and inhibition<sup>[17–19]</sup>. The current RT-PCR results demonstrated that gene expression levels of p15 and caspase-3 p17 were significantly increased in a dose-dependent manner in cells treated with crotoxin for 48 h. This suggests that crotoxin may induce Eca-109 cell apoptosis via caspase-3 signaling pathways.

Eca-109 human esophageal carcinoma cells were inoculated into nude mice to establish a tumor transplant model, and crotoxin was found to inhibit the growth of the transplanted tumors. Western blotting demonstrated that p15 and caspase-3 p17 protein levels were increased in tumors treated with crotoxin, while Bcl-2 protein was significantly reduced. There were no obvious adverse effects associated with tumor inhibition, and weight, mood, diet, and activity were all similar in the crotoxin and control groups.

## Conclusions

*Crotalus durissus* neurotoxin (crotoxin) inhibits the growth of esophageal carcinoma Eca-109 cells *in vitro* via a mechanism involving apoptosis induction and a  $G_1$  phase block. Apoptosis induction may, in turn, be related to caspase-3 activation. Local crotoxin treatment can inhibit the growth of subcutaneously transplanted esophageal Eca-109 tumors in nude mice, and this inhibition may be related to the upregulation of p15 and caspase-3 p17, as well as the downregulation of Bcl-2 protein expression.

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## Author contribution

Jing-kang HE and Yan XIE designed research; Xiang-sheng WU performed research; Xiang-sheng WU and Yan WANG wrote the manuscript; Zheng-hong QIN and Yan WANG edited the manuscript; Yan WANG and Rong HAN analyzed data.

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