

# Serum from rabbit orally administered cobra venom inhibits growth of implanted hepatocellular carcinoma cells in mice

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## Abstract

**AIM:** To investigate the inhibitory effect of serum preparation from rabbits orally administered cobra venom (SRCV) on implanted hepatocellular carcinoma (HCC) cells in mice.

**METHODS:** An HCC cell line, HepA, was injected into mice to prepare implanted tumors. The animals ( $n=30$ ) were divided randomly into SRCV, 5-fluorouracil (5-FU), and distilled water (control) groups. From the second day after transplantation, 20 mg/kg 5-FU was administered intraperitoneally once a day for 9 days. SRCV (1 000 mg/kg) or distilled water (0.2 mL) was given by gastrogavage. Tumor growth inhibition was described by the inhibitory rate (IR). Apoptosis was detected by transmission electron microscopy (TEM), flow cytometry (FCM), and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL). Student's *t*-test was performed for statistical analysis.

**RESULTS:** The tumor growth was inhibited markedly by SRCV treatment compared to that in the control group ( $P<0.01$ ). The treatment resulted in a significant increase in the apoptotic rate of cancer cells by the factors of  $10.5\pm2.4\%$  and  $20.65\pm3.2\%$  as demonstrated through TUNEL and FCM assays, respectively ( $P<0.01$ ). The apoptotic cells were also identified by characteristic ultrastructural features.

**CONCLUSION:** SRCV can inhibit the growth of implanted HepA cells in mice, and the apoptosis rate appears to elevate during the process.

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## INTRODUCTION

Snake venoms are complex mixtures of pharmacologically active polypeptides, some are of potential therapeutic value

for embolism, cancer and other severe human disorders. Several snake venoms and their components have been demonstrated to be able to inhibit tumor growth and to induce apoptosis of neoplastic cells *in vitro* and *in vivo*<sup>[1-7]</sup>. We prepared a serum preparation from rabbits administered cobra venom (SRCV)<sup>[8]</sup>. Our *in vitro* studies have shown its growth-inhibitory and apoptosis-inducing effects of SRCV on cancer cells<sup>[9]</sup>. In the present study, we observed its effects *in vivo* using implanted hepatocellular carcinoma (HCC) cell line HepA in mice.

## MATERIALS AND METHODS

### Drugs and reagents

5-FU was purchased from Nantong Pharmaceutical Co (Cat. No. 001121; Nantong, Jiangsu, China). SRCV was prepared as described previously<sup>[8]</sup>. Briefly, the rabbits were given oral Chinese cobra (*Naja naja atra*) venom 45 mg/kg (Guangzhou Research Institute of Snake Venom, Guangzhou, Guangdong, China) once a day for 3 days. Serum was collected from the rabbits at 4 h after the last administration, then heated in a water bath for 30 min at 56 °C, frozen at -20 °C, lyophilized using a vacuum drier and stored at 4 °C.

### Animals

Female Kunming mice (18-22 g, No. 26-2002A002) were supplied from the Medical Animal Center of Guangdong Province. All animals were fed on basic diet and water. The cell line HepA was provided by the Cancer Institute of Sun Yet-Sen University.

### Experimental schedule

As described previously<sup>[10,11]</sup>, HepA cells,  $2\times10^7$ /mL, were injected subcutaneously into mice, 200  $\mu$ L for each. Thirty Kunming mice with implanted HepA tumor were divided randomly into SRCV-, 5-FU-treated groups and control group. From the second day after the implantation, 20 mg/kg 5-FU was administered intraperitoneally once a day for 9 days. SRCV (1 000 mg/kg) or distilled water (0.2 mL, control group) was given by gastrogavage. The mice were sacrificed at 24 h after the last administration. The tumors were isolated and weighed immediately in order to calculate the inhibitory rate (IR) according to the formula: IR of tumor (%) =  $(1 - \text{tumor weight in test groups} / \text{mean tumor weight in control group}) \times 100\%$ . Then, the tumors were fixed and used for transmission electron microscopy (TEM), flow cytometry (FCM) and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL). All of the tests were repeated twice.

### Morphological analysis of apoptosis

Dissected tumor samples were fixed with 2.5 % glutaraldehyde for 1 h. After washed three times in a buffer, the samples were post-fixed in 1 % OsO<sub>4</sub> in a cacodylate buffer for 1 h, then dehydrated in graded ethanol and embedded in epoxy resin (Agar 100). Thin sections (70 nm) were stained with uranyl acetate and reynolds lead citrate and examined at 75 kV in an electron microscope (JEM-100CX 11/T). Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by TEM.

### Flow cytometry analysis

Cell apoptotic rate was quantitatively determined by flow cytometry. The percentage of cells with a sub-G1 DNA content was taken as the fraction of apoptotic cell population<sup>[12,13]</sup>. According to the procedure described previously<sup>[14-16]</sup>, tumor tissues were sliced at a thickness of 400-500  $\mu\text{m}$ , then the slices were gently pulverized using a mortar and pestle in phosphate-buffered saline (PBS) (pH7.2). The cell suspension was infiltrated through 200 and 350  $\mu\text{m}$  meshes to remove residues. The cells were collected by centrifugation at 2 000 rpm for 10 min. The cell suspension was fixed in 70 % ice-cold ethanol in PBS, and stored at -20 °C. Prior to analysis, the cells were washed and resuspended in PBS, then incubated with 10 mg/mL RNase A for 3-5 min and 50  $\mu\text{g/mL}$  propidium iodide (PI) at 4 °C for 30 min in a dark chamber. The apoptotic cells having DNA strand breaks that had been labeled were detected by a flow cytometer (FACSCan, Becton Dickinson, San Jose, California, USA).

### TUNEL reaction

An ApopTag plus peroxidase *in situ* apoptosis detection kit (Intergen Co Ltd., Burlington, Massachusetts, USA) was used to visualize the cells with DNA fragmentation. The procedure was performed following instructions of the manufacturer and in reference of the previous observations<sup>[17-19]</sup>. Briefly, 4- $\mu\text{m}$  thick sections were dewaxed and hydrated, treated with 20  $\mu\text{g/mL}$  proteinase K for 15 min at 37 °C, equilibrated in a buffer for 5 min at room temperature, and incubated in a buffer containing terminal deoxynucleotidyl transferase (TdT) enzyme for 1 h in a humidified chamber at 37 °C. The reaction was demonstrated by incubation with anti-digoxigenin-peroxidase for 30 min in a humidified chamber at room temperature and visualized in a buffer containing diaminobenzidine (DAB).

The positive cells were identified, counted and analyzed based on morphological characteristics of apoptotic cells as previously described<sup>[17]</sup>. Under the light microscope, apoptotic cells manifested as brownish staining in the nuclei. Non-necrotic zone was selected in the tissue section and images were randomly selected. At least 1000 tumor cells were counted, and the percentage of TUNEL-positive cells was determined.

### Statistical analysis

The data shown were mean values of 8-10 samples and expressed as mean  $\pm$  standard deviations. Student's *t*-test was performed for statistical analysis. A *P* value less than 0.05 was considered statistically significant.

## RESULTS

### Anti-tumor effect of SRCV on implanted HepA tumor

In two separate experiments, the IRs were 30.4 % and 35.8 % after treatment with SRCV. The data, listed in Table 1, demonstrated the inhibitory effect of SRCV treatment on implanted HepA tumor growth, though it was not as strong as that of 5-FU.

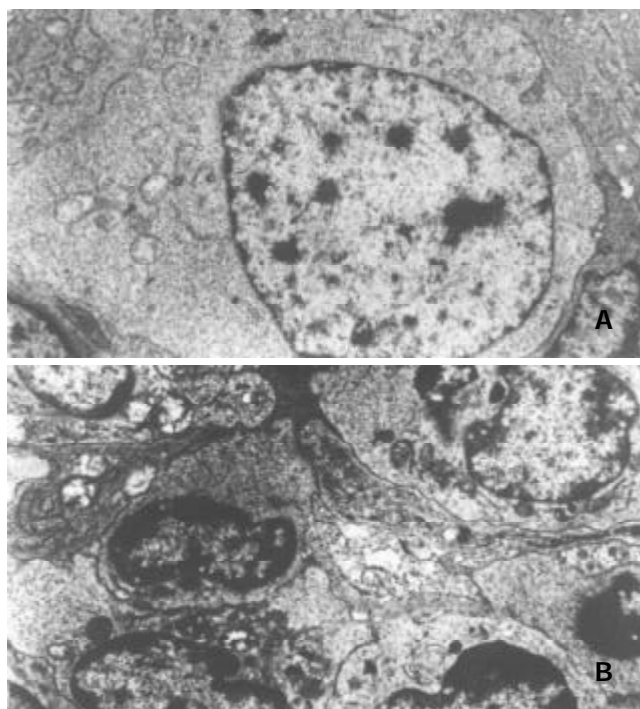
**Table 1** Anti-tumor effect of CVSR on implanted HepA tumors in mice ( $n=10$ ,  $\bar{x}\pm s$ )

Group	Dose	Tumor weight/(g)		Inhibition rate (%)	
		First	Twice	First	Twice
SRCV	1 000 mg/kg	1.14 $\pm$ 0.28 <sup>a</sup>	1.14 $\pm$ 0.13 <sup>a</sup>	30.35 $\pm$ 17.07	35.83 $\pm$ 7.11
5-FU	20 mg/kg	0.78 $\pm$ 0.14 <sup>a</sup>	0.99 $\pm$ 0.22 <sup>a</sup>	52.57 $\pm$ 8.56	44.39 $\pm$ 10.5
Control	0.2 ml	1.63 $\pm$ 0.26	1.78 $\pm$ 0.47		

<sup>a</sup>*P*<0.01 vs control group.

### Apoptosis - inducing effect of SRCV in mice with implanted HepA tumor

The apoptosis-inducing effect of SRCV was confirmed by electron microscopy. Compared with control group (Figure 1A), morphological changes indicative of apoptosis included cell shrinkage, nuclear chromatin condensation and peripheral shift of condensed chromatin to nuclear membrane or formation of crescent. In addition, the nuclear membrane was intact and there was little or no swelling of mitochondria or other organelles (Figure 1B).



**Figure 1** Morphological changes characteristic of apoptotic cells under transmission electron microscope in implanted tumors after treatment with SRCV. A: The normal tumor cells in distilled water control group ( $\times 5000$ ). B: The apoptotic cells in SRCV group ( $\times 5000$ ).

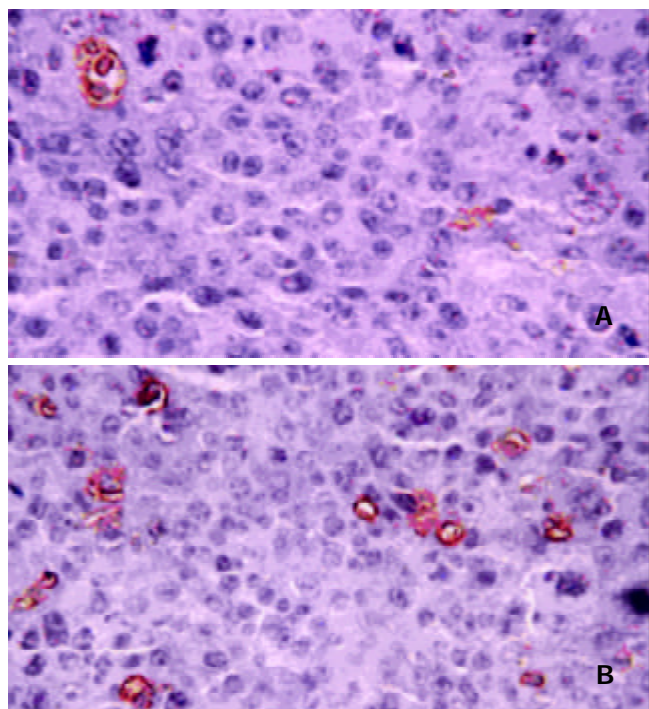
**Table 2** Apoptotic rates (AR) of implanted tumor in SRCV treated mice determined by TUNEL and FCM methods ( $n=8$ ,  $\bar{x}\pm s$ )

Groups	Mean apoptotic rate $\pm$ SEM (%)	
	TUNEL	FCM
SRCV	10.50 $\pm$ 2.42 <sup>b</sup>	20.65 $\pm$ 3.2 <sup>b</sup>
Control group	1.67 $\pm$ 1.3	2.28 $\pm$ 1.54

<sup>b</sup>*P*<0.01 vs control group.

The apoptosis-inducing effect of SRCV was further confirmed by FCM and TUNEL. After treatment with SRCV, the apoptotic rate was significantly increased in the SRCV group compared with the control group (Table 2). In TUNEL

assay, induction of apoptosis was represented by an increase in DNA fragments detected by a peroxidase reaction (Figure 2A), and the apoptotic cells in control tumors were scarcely scattered (Figure 2B).



**Figure 2** Apoptotic cells of implanted HepA tumors in SRCV treated mice detected by TUNEL assay. A: control group ( $\times 400$ ), B: SRCV 1 000 mg/kg group ( $\times 400$ ).

## DISCUSSION

Snake venoms have inhibitory effects on the growth of a variety of tumors *in vitro* and *in vivo*<sup>[20,21]</sup>. Markland *et al.* found that contortrostatin (CN), a homodimeric disintegrin from southern copperhead venom, inhibited dissemination ovarian cancer in a nude mouse model<sup>[22]</sup>. According to Da Silva *et al.*, *Bothrops jararaca* venom (BjV) had anti-tumor effects on Ehrlich ascites tumor (EAT) cells *in vivo* and *in vitro*<sup>[23]</sup>.

Snake venom was also shown to induce apoptosis in tumors. Apoptosis, in contrast to necrosis, was an active process of gene-directed cellular suicide<sup>[24]</sup>. It has been clear that apoptosis is often upregulated in tumor by many anticancer drugs<sup>[25-32]</sup>.

Since Araki *et al.* first described that hemorrhagic snake venoms induced apoptosis in vascular endothelial cells (VEC)<sup>[33]</sup>, data have been accumulated rapidly about apoptosis-inducing action of various snake venoms and their active components. In 1994, Strizhkov *et al.* reported that both neurotoxin II (NT II) from venom of *Naja naja oxiana* and 20-30 kDa proteins partially purified from pig brain (NTIm) cross-reacting with antibodies to NT II were cytotoxic to L929 and K562 tumor cells at concentrations of  $10^{-6}$ - $10^{-8}$  M, and induced apoptosis in L929 and K562 cells *in vitro*<sup>[34]</sup>. After that, L-amino acid oxidase (LAO) was found to induce apoptosis in human embryonic kidney cell line 293T<sup>[35]</sup> and human monocyte line MM6<sup>[36]</sup>. Recently, Araki *et al.*<sup>[37]</sup> and Masuda *et al.*<sup>[38]</sup> associated integrins with vascular apoptosis-inducing protein 1 (VAP1)-induced apoptosis. Data from Zhao *et al.* indicated that snake venom secreted phospholipase A<sub>2</sub> (sPLA<sub>2</sub>) induced apoptosis in Mv1Lu cells in a dose- and time-dependent manner, and was associated with a rapid increase in intracellular ceramide level<sup>[39]</sup>.

The clinical trial using snake venom and their active components have succeeded in cancer therapies, but its

application was confined to the auxiliary treatment of patients in the late stage<sup>[40,41]</sup>. Its toxic and side effects were unavoidable. Great efforts have been made<sup>[42-45]</sup>, but the problem remains unresolved. So a long-standing goal in snake venom therapy of cancer is to find a stable, low toxic, highly effective chemotherapeutic agent that selectively targets tumor cells. Based on this idea, we prepared the SRCV agent<sup>[8]</sup>.

In our previous studies, the anti-tumor activity and apoptosis-inducing effect of SRCV were shown *in vitro* using HepG-2, HL-60 and human lung adenocarcinoma cell line, and no cytotoxicity was observed on human fetal lung fibroblast cells<sup>[46-48]</sup>. The results presented herein demonstrate that SRCV has inhibitory and apoptosis-inducing effects on implanted HepA tumors. Further studies are needed to identify the active components of SRCV and to elucidate their underlying mechanism.

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