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Abstract: This study was designed to investigate the effect of a seashell protein Haishengsu (HSS) on the immuno logical function in mice with Ehrlich ascites tumor. Ehrlich ascites tumor-bearing mice were divided into three HSS groups (25, 50 and 100mg/kg, i.v., respectively), cyclophosphamide (10mg i.p.) and control group. The immunological function was assessed by measuring the phagocytizing capacity of the peritoneal macrophages and neutrophils, as well as the number of spleen hemolytic plaque-forming cells. The percentage of blood T-lymphocytes was also evaluated. The number and the percentage of phagocytizing macrophages and neutrophils in the 50 and 100mg/kg HSS groups were higher than in the control and the cyclophosphamide groups (P<0.01). The hemolytic plaque-forming cells in the three HSS groups (10.8-±1.2, 16.9-±3.9 and 25.3-±2.9, respectively), was greater than in the control (7.3-±1.4), or the cyclophosphamide group (0.33-±0.4) (all P<0.01). In all HSS groups, the percentage of blood T3, T4 and T8 was higher than in the cyclophosphamide and the control group (all P<0.01). We conclude that HSS has significant immune-modulating effect in mice with Ehrlich ascites tumor.

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Antitumor effect of a seashell protein Haishengsu on Ehrlich ascites tumor: an experimental study

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Running title: Haishengsu and Ehrlich ascites tumor

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Abstract

To investigate the *in vivo* effect of a seashell protein Haishengsu (HSS) on Ehrlich ascites tumor, mice were inoculated with Ehrlich ascites tumor cells. They were randomly divided into three HSS groups and control group. The survival times in the three HSS-treated groups was longer than in the control ($P<0.01$) and the increased life span in the high dose HSS group was greater than in the lower dose groups ($P<0.05$). In comparison with control group, the mice receiving pretreatment of HSS had longer survival times and greater life spans following inoculation of the ascities tumor ($P<0.05$). In conclusion, HSS prolongs survival times and increases the life spans of Ehrlich ascites tumor bearing mice. Pre-treatment with HSS also diminishes the detrimental effect of Ehrlich ascites tumor on the prognosis of these animals.

Key words: Ehrlich ascites tumor; mice; haishengsu; cyclophosphamide; cancer

Introduction

Tegillarca granosa L. (order Arcoida) has been used as a traditional Chinese medicine in mainland China to treat a variety of human cancers for more than a century. *Haishengsu* (HSS) is a protein purified from *Tegillarca granosa*. It has a molecular weight of approximately 15 kDa but an unknown chemical structure. HSS was found to have a potent suppressive effect on several types of tumor cells *in vivo* and *in vitro* [1-3].

The *in vivo* anticancer effects of HSS were investigated in two small clinical trials. Daily intravenous administration of 2.4 mg HSS for 4 weeks resulted in complete or partial remission of non-small-cell lung cancer in 49% of the patients [4]. A similar remission rate was found in patients with renal cancer [4]. There were no significant adverse effects in patients receiving the 4-week HSS treatment [4]. In a more recent randomized, double-blind, placebo-controlled trial, HSS treatment was associated with an increased remission rate in the patients with non-small-cell lung cancer [5].

The primary purpose of this study was to investigate the *in vivo* effect of HSS on Ehrlich ascites tumor. Both the preventive and therapeutic effects of HSS were evaluated.

Materials and methods

Sources of equipment, drugs, cells and animals

This study was approved by the institution review board of Liaocheng People's Hospital. The manufacturers of the major equipment and drugs were as follow: Fluorescence

microscope (XSZ-H52 y1) - Chongqing Optical Instrument Corporation (China); high-speed micro-centrifuge (LG15-W) - Beijing Medical Centrifuge Plant (China); electric constant temperature incubator (28YX-1) - Weifang Medical Instrument Corporation (Shandong, China)

HSS was purchased from Qingdao Haisheng Oncology Hospital (Shandong, China, batch number 990211) and was dissolved in phosphate buffer saline for iv injection. Ehrlich ascites tumor cells were provided by the Chinese Academy of Sciences (Shanghai, China). The Animal Center of Shandong Medical University (Jinan, China) provided the mice (weight 18-22 g).

Effect of HSS on Ehrlich ascites tumor

Tumor cells were aspirated and suspended in phosphate buffer saline. Cell viability was tested by staining with trypan blue prior to the experiment. The Ehrlich ascites tumor cells were diluted with saline (ratio 1:4). Under sterile conditions, healthy mice were inoculated intraperitoneally with 0.2 ml of the diluted tumor cell solution. Abdominal tumors were palpable three days after the inoculation.

Twenty-four hours after the inoculation, the mice were randomly divided into 5 groups (10 animals in each group): three HSS groups (100, 700 and 1000 mg/kg group, iv, once daily for 10 days); cyclophosphamide (10mg/kg, iv, once daily for 5 days) and the control group (normal saline, iv, once daily for 10 days).

The selection of HSS doses in this protocol were based on a pilot study where the minimum effective dose of HSS in suppressing tumor growth was approximately 100

mg/kg.

The mice were closely monitored after the drug administration. The time of death and days of the life were recorded, and the increased life span (ILS) was calculated, using the following formula [6]: *ILS* (%) = (Average survival time of the treated group-average survival time of the control group)/average survival time of the control group × 100%.

Preventive effect of HSS

Fifty mice were randomly divided into three HSS groups (147, 210 and 300 mg/kg), cyclophosphamide (10mg/kg, iv, once daily for 5 days) and control group. Drug administration and the course of therapy were the same as described above. The doses of HSS in this protocol were based on a pilot study where the minimum effective dose of HSS in preventing the tumor cell growth was approximately 147 mg/kg.

The mice were inoculated with Ehrlich ascites tumor cells immediately after the 10-day therapy with HSS. The time of death and days of the life were recorded, and the life span was calculated as described above.

Effect of HSS on the inoculation capacity of Ehrlich ascites tumor cells

Fifty healthy mice were inoculated with the Ehrlich ascites tumor cells. Twenty-four hours after the inoculation, they were randomly divided into three HSS groups (147, 210 and 300 mg/kg, respectively), cyclophosphamide (10 mg/kg) and the control group. Three days after the treatment, the peritoneal fluid of the mice was collected from each

group. The fluid was diluted with normal saline (ratio 1:6), and injected intraperitoneally (0.2 ml) into 40 healthy mice (three HSS and one control group, 10 animals each). The time of death and days of life were recorded, and the life span was calculated.

Statistical analysis

Data were expressed as means ± SD. SAS6.12 software was used for data analysis. Numerical data were analyzed with one-way ANOVA followed by multiple comparison (LSD) test. Categorical data between two groups were analyzed with Chi-square test. $P<0.05$ was considered statistically significant.

Results

Antitumor effect

In the control group, there was a significant increase in the average body weight due to ascites and tumor growth (Table 1). The body weight of the HSS 490 and 700mg/kg groups was similar to that in the control group. But in the HSS 1000mg/kg, the average body weight was lower than in the control group (Table 1, $P<0.05$).

There was no significant difference in the surviving times between the cyclophosphamide and the control group ($P>0.05$). The average survival times of the HSS groups were significantly longer than in the control or the cyclophosphamide group (Table 1, $P<0.05$ or 0.01). The survival time in the HSS 1000 mg/kg was longer than in the 490 mg/kg group ($P<0.05$). The increased life span of the HSS 1000 mg/kg

group was greater than in the 490 or 700 mg/kg groups (P<0.05).

Preventive effect

Table 2 shows the survival times and life spans in mice receiving HSS before undergoing ascites tumor cell inoculation. The survival times in the three HSS groups were longer than in the control or the cyclophosphamide group after tumor cell inoculation (P<0.05). The increased life span of the HSS 300 mg/kg group was greater than in the 147 mg/kg group (P<0.05).

Effect on the inoculation capacity of Ehrlich ascites tumor cells

Table 3 shows the prognosis in mice inoculated with ascites tumor cells from mice had already been treated with HSS. The body weight in the HSS 300 mg/kg group was lower than in the control group (P<0.05). The survival times in the three HSS groups were longer than in the control group (P<0.05). The increased life span of the HSS 300 mg/kg group was greater than in the HSS 147 mg/kg group (P<0.05).

Discussion

The major findings of this study are: 1) HSS dose-dependently prolonged the survival time and increased the life span of mice inoculated with Ehrlich ascites tumor; 2) Pre-treatment with HSS for 10 days diminished the detrimental effect of ascites tumor on the survival times and life span; 3) Ehrlich ascites tumor cells from HSS-treated mice had a significantly reduced inoculation capacity; 4) cyclophosphamide (10 mg/kg,

i.v.) was not effective in suppressing or preventing Ehrlich ascites tumor in this experimental protocol.

We evaluated the *in vivo* antitumor activity of HSS by using a standard mice model. In the treatment and preventive protocols, HSS was administered intravenously at three different doses to establish the dose-dependent responses. When they were used after the tumor inoculation, all three HSS doses extended the survival times as well as the life span of the tumor-bearing mice. These results indicate that HSS may be used as an anticancer agent for the management of ascites tumor.

The other finding of potential importance is the preventive effect of HSS. When healthy mice were pre-treated with HSS, subsequent inoculation with ascites tumor cells was less effective in shortening the life span than in the mice receiving no pre-treatment with HSS. These results raise the possibility that HSS may be used preventively in cancer management.

It is unclear what molecular or cellular mechanisms are involved in the antitumor effect of HSS as observed in this study. HSS was found to inhibit tumour cell line Ketr-3 and A549, blocking the cell cycle at phase G0/G1 and G2/M [7]. It suppressed the growth of S180 tumor cells in rats by up to 55% [7]. Our recent study found that HSS suppressed the growth of leukemia K562 cell by inhibiting the G0/G1 and S phases of the cell cycle [3]. HSS also induced apoptosis in these leukemia cells by reducing the expression of apoptosis suppressor bcl-2, and increasing the expression of apoptosis promoting bax [3]. Whether induction in apoptosis or inhibition of cell growth

has contributed to the effect of HSS on the Ehrlich ascites tumor needs further investigation.

In conclusion, intravenous administration of a seashell protein HSS for 10 days is associated with significant survival benefit in mice affected by Ehrlich ascites tumor. HSS is able to dose-dependently prolong the survival times and increase the life span by up to 50% in this animal model.

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Table 1. The antitumor effect of Haishengsu (HSS).

Groups	Body weight (g)		Survival time	ILS
	Before	After	(days)	
Control	20.5±0.8	30.7±0.6	11.30±8.1	
HSS (490 mg/kg)	19.8±0.5	29.4±0.6	14.37±2.2*	27.2%
HSS (700 mg/kg)	19.2±0.5	28.2±1.6	15.83±2.3*	40.1%
HSS (1000 mg/kg)	20.7±0.7	25.6±2.8*	17.29±2.9**	53.0%$^{\#}$
Cyclophosphamide (10 mg/kg)	20.8±0.7	20.7±0.7$^{\#\#}$	12.70±1.2	12.4%

Note: 10 animals in each group. *P<0.05, **P<0.01 HSS *vs* control or cyclophosphamide. ILS: increased life span. $^{\#}$: P<0.05 compared with HSS 700 and 490 mg/kg groups.

Table 2. The preventive effect of Haishengsu (HSS) on Ehrlich ascites tumor.

Group	Body weight (g)		Survival time	ILS
	Before	After	(days)	
Control	19.8±1.2	25.4±4.2	11.9±4.6	
HSS (147 mg/kg)	18.9±0.9	23.8±2.9	13.5±2.2*	33.7%*
HSS (210 mg/kg)	18.8±0.7	22.6±1.6	14.1±2.0*	39.4%**
HSS (300 mg/kg)	19.0±0.5	21.4±2.5*	15.0±2.5*	48.5%**[#]
Cyclophosphamide (10 mg/kg)	18.7±1.1	18.2±3.1	10.1±2.3	17.7%

Note: 10 animals in each group. *P<0.05, **P<0.01 HSS *vs* control or cyclophosphamide. ILS: increased life span. [#]: P<0.05 compared with HSS 147 mg/kg group.

Table 3. Survival time and life span of mice inoculated with ascites tumor cells from mice pretreated with Haishengsu (HSS).

Group	Body weight (g)		Survival	ILS
	Before	After	time(days)	
Control	18.6±0.4	26.4±1.7	12.2±4.1	
HSS (147 mg/kg)	18.3±0.9	24.3±2.1	15.60±1.9*	40.5%
HSS (210 mg/kg)	18.4±0.2	22.7±1.8	16.50±2.1*	48.7%
HSS (300 mg/kg)	18.2±0.7	21.3±1.2*	17.50±1.8**	57.7%[#]
Cyclophosphamide (10 mg/kg)	19.1±1.1	20.3±1.2	11.1±2.1	10.3%

Note: 10 animals in each group. *P<0.05, **P<0.01 HSS *vs* control or cyclophosphamide. ILS: increased life span. [#]: P<0.05 compared with 147 mg/kg group.