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Immune Effect of Stichopus Japonicus Acid Mucopolysaccharide on Hepatocarcinoma22-bearing Mouse*

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ABSTRACT Objective: As a natural biologically active substance, Stichopus japonicus acid mucopolysaccharide (SJAMP) has significant anti-tumor effect. The present study aimed to investigate the immune effect of SJAMP on Hepatocarcinoma22-bearing mouse. **Methods:** The mice were injected H22 cells into subcutaneous of the right armpit, then were randomly divided into model group, 5-FU group and SJAMP intervention groups(group A, B, C). Three doses of SJAMP (6.25 mg/kg, 12.5 mg/kg, and 25 mg/kg administered once a day via intraperitoneally inject) were given to mice continuously for 12 days. Blood samples were drawn by excising eyeball and then all the mice were sacrificed by cervical dislocation. The spleens and thymuses were removed under sterile conditions, and the spleen indexes and thymus indexes were calculated. Neutral red method was used to detect macrophage phagocytosis. Spleen Lymphocytes proliferation capability was assayed by CCK-8 method and TNF-α was detected by ELISA method. **Results:** SJAMP treatment significantly inhibited Hepatocarcinoma22 by reducing the weight of tumor nodules. SJAMP administration also improved spleen indexes and thymus indexes, abdominal macrophage phagocytosis and spleen Lymphocytes proliferation activity. Furthermore, SJAMP groups showed a decrease in serum level of TNF-α. **Conclusion:** SJAMP effectively inhibits the growth of tumor through stimulating the immune organs proliferation and enhancing immunity ability of Hepatocarcinoma22-bearing mouse. The results provide an experimental and theoretical basis for the study of the anti-tumor effects of SJAMP.

Key words: Stichopus japonicus acid mucopolysaccharide; Hepatocarcinoma22; Immune function

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Introduction

WHO International Cancer Research Center estimates that Chinese Hepatocellular carcinoma incidence and mortality rate all exceed half of the world[1], which seriously endangers the health of residents. Poor selectivity of chemotherapy drugs makes it difficult to avoid damage to normal tissues and organs, especially to the immune system. Enhancing the host's immune response is considered as a possible mean to bate tumor without harm to the host. Sea cucumber has been valued for hundreds of years in the Chinese diet as a food delicacy, as well as a medicine for a wide variety of diseases. In the United States and Canada, sea cucumber tissues are used as nutraceuticals for over-the-counter dietary health supplements [2]. SJAMP is extracted from the body wall or visceral of sea cucumber. Studies have shown SJAMP has multiple pharmacologic properties, including anticoagulant, immunologic regulation, antiviral properties [3], and broad-spectrum anti-tumor [4]. However, the mechanism of anti-tumor activity of SJAMP remains to be explored. There is a close relationship between the immune state and the progression of tumors [5]. Therefore, this study aims to investigate the inhibition on tumor and the immune enhancement in mouse of SJAMP.

1 Materials and methods

1.1 Materials

50 SPF Kunming mice, Male and female each half, 18-22 g, (purchased from Shan dong lukang Pharmaceutical Co.,Ltd., quality certification number: SLXK Lu 20081022); H22 cell, YAC-1, Shandong Academy of Medical Sciences provided; SJAMP, Ocean University of China School of Food Science and Engineering extracted; RPMI-1640 medium, 0.25 % Trypsin-EDTA digestion (the United States Hyclone Company); Cell proliferation and cytotoxicity assay kit(CCK-8), (Beijing solarbio S&T Co.,Ltd.); Lymphocyte separation medium, Neutral red, (Solarbio company); ELISA Kit, (Dobio Biology Technology Inc.).

1.2 Animal Model

The mice were injected H22 cells into subcutaneous of the right armpit[6] after one week feeding adaptation and were randomly divided into model group, 5-FU group and SJAMP intervention groups(group A, B, C).There were 10 mice in each group. After 24 hours, 5-FU group received 5-FU (20 mg/kg) and SJAMP intervention groups received different doses of SJAMP (respectively

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6.25 mg/kg, 12.5 mg/kg, 25 mg/kg) by intraperitoneal injection once a day continuously for 12 days. Meanwhile, the model group was injected with equivalent saline. The mice were fed with standard laboratory diet and water. Blood samples were drawn by excising eyeball 24 hours after the last administration and then all the mice were sacrificed by cervical dislocation. The spleens and thymuses were removed and weighted under sterile conditions. The tumors were took off and weighted.

The tumor inhibition rate(%)=(model group tumor weight-intervention group tumor weight)/model group tumor weight× 100%

The spleen index=spleen weight(mg)/body weight(g)

The thymus index=thymus weight(mg)/body weight(g)

1.3 The proliferation ability of spleen lymph cell

The spleens were washed with PBS solution two times and were grinded in sterilized 200-mesh net while rinsing with PBS solution [7]. Washed spleen cells suspension with PBS solution 2 times, then 1000 r/min centrifuged for 10 minutes, adjusted the cell concentration to 2× 10^6/ml. Three holes were set in 96-well plate each sample, 100 μL cell suspension and 100 μL ConA were added to every hole, then incubated in 37℃ 5%CO_2 humidified incubator for 48 hours, added CCK-8 10 μL in each well, incubated in 37℃ 5 % CO_2 humidified incubator for 4 hours then measured the A value at 450 nm.

1.4 Detect macrophage phagocytosis phagocytosing neutral red method

This assay was performed as described previously [8]. Five milliliters of PBS was injected into the peritoneal of the mice that were sacrificed by cervical dislocation. Abdomens of the mice were kneaded one minute. The lavage fluid was collected and cell concentration was adjusted to 2× 10^6/ml. Each sample was inoculated this cell suspension 150 μL into 96-well plates and cultured for 4 hours in 37℃ 5 % CO_2 humidified incubator. The culture medium and not adherent cells were sucked out. Then added fresh culture medium each well, incubated the plates in 37 ℃ 5 % CO_2 humidified incubator. Ten hours later, 50 μL 3 mg/ml neutral red solution was added into each well, and the plates were incubated in 37 ℃ 5 % CO_2 humidified incubator for 4 hours.Each well was washed three times with PBS and 150 μL lysis buffer (0.1 mol/L acetic acid:ethanol=1:1) was added in each well. The OD values were detected at 492 nm with enzyme-linked immunosorbent tester.

1.5 Observe serum TNF-α

This experiment was operated in accordance with the ELISA kit instruction. The absorbance value of each well was detected in enzyme-linked immunosorbent tester at 450nm. The production of TNF-α in the sample was calculated according to the standard curve.

1.6 Data Processing

The data were statistically analyzed using one-way analysis of variance (ANOVA). Post-hoc comparisons were carried out by the least-significant difference (LSD) test. All data were expressed as the means± SD. Probability values lower than 0.05 were considered significant.

2 Results

2.1 Growth conditions of mice in the feeding process

The growth of the mice in model group and SJAMP groups was in good condition and body weight increased rapidly as well as gloss coat and normal urine. The mice in 5-FU group appeared different levels of apathetic, dull coat, gloss loss, diarrhea, loss of appetite, reduced activity, slow growth and weight loss. The growth state of mice in group B and C was significantly better than in 5-FU group. High dose of SJAMP and 5-FU significantly inhibited tumor growth (P<0.05) (Table 1).

Table 1 The effect of inhibition of SJAMP on H22 liver cancer in mouse($\bar{x}$± s)

2.2 Thymus index,spleen index

Groups	Number (n)	Tumor weight ($\bar{x}$± s)	The tumor inhibition rate(%)
Modal group	10	1.30± 0.59	_
5-FU group	9	0.58± 0.34[a]	55.53
SJAMP-A group	10	1.10± 0.36[b]	15.38
SJAMP-B group	10	0.95± 0.55	27.06
SJAMP-C group	10	0.69± 0.53[a]	47.23

Note: a: P<0.05, vs modal group.

The thymus indexes in SJAMP intervention groups were higher than in 5-FU group (P<0.05) and the thymus indexes in 5-FU group were lower than in modal group (P<0.05); The spleen indexes in SJAMP-B group and SJAMP-C group were higher than in modal group, 5-FU group, and SJAMP-A group (P<0.05), but the spleen indexes in 5-FU group were lower than in modal group (P>0.05)(Table 2).

Table 2 The impact of SJAMP on spleen index and thymus index of H22 cancer mouse($\bar{x}$± s)

2.3 Abdominal macrophage phagocytosis of neutral red

Groups	Number (n)	Thymus index	Spleen index
Modal group	10	3.00± 1.45	6.73± 2.36
5-FU group	9	1.14± 0.53[a]	5.75± 1.93
SJAMP-A group	10	2.25± 0.63[b]	8.46± 2.72
SJAMP-B group	10	2.26± 1.29[b]	13.01± 5.58[abc]
SJAMP-C group	10	2.19± 1.18[b]	13.58± 4.44[abc]

Note: [a]; P<0.05, vs modal group; [b]; P<0.05, vs 5-FU group; [c]: P<0.05, vs SJAMP-A group.

Compared with that in model group, phagocytosis macrophages in 5-FU group declined significantly (P<0.05), while SJAMP-C groups showed greater macrophage phagocytic capacity (P<0.05). Compared with that in 5-FU group, SJAMP groups

showed greater macrophage phagocytic capacity (P<0.05). The intervention dose and phagocytic activity showed a dose-response relationship(Table 3).

2.4 The proliferation ability of spleen lymph cell

Compared with that in 5-FU group, spleen lymph cells in SJAMP groups showed greater proliferation ability (P<0.05), and the intervention dose and proliferation activity showed a dose-response relationship(Table 3).

2.5 The impact of SJAMP on TNF-α($\bar{x}\pm s$)

Compared with that in modal group, TNF-α in 5-FU group and SJAMP groups was lesser (P<0.05), and SJAMP-C group was lower than 5-FU group (P<0.05) (Table 3). The SJAMP groups showed no dose-response relationship (P>0.05), which may be related with small sample size (Table 3).

Table 3 The impact of SJAMP on macrophage phagocytosis of neutral red,the proliferation ability of spleen lymph cell and the serum level of TNF-α($\bar{x}\pm$ s)

Group	Number (n)	Phagocytosis (OD value)	Proliferation (OD value)	TNF-α (OD value)
Modal group	10	0.672± 0.152	0.524± 0.200	65.81± 4.41
5-FU group	9	0.465± 0.081[a]	0.395± 0.207	54.14± 4.70[a]
SJAMP-A group	10	0.769± 0.163[b]	0.661± 0.296	57.80± 9.86[a]
SJAMP-B group	10	0.817± 0.171[b]	0.818± 0.127[ab]	52.92± 5.26[a]
SJAMP-C group	10	0.967± 0.193[abc]	0.871± 0.108[abc]	51.35± 10.97[ab]

Note:[a]; P<0.05, vs modal group;[b]; P<0.05, vs 5-FU group;[c]: P<0.05, vs SJAMP-A group.

3 Discussion

Tumor development closely relates to the immune situation of organism [9]. When tumor growth in progressive, the immune function is suppressed [10,11]. Meanwhile, multifarious immune mechanisms of anti-tumor effect occur in the body [12]. Generally, Immune cells play a major role in anti-tumor immune function[13].

Thymus and spleen are important organs involved in the immune response, and their development directly relate to the immune function of the organism. Spleen index and thymus index reflect the immune status of the body to a certain extent [14]. The present study shows that SJAMP increases spleen index and maintain thymus index of H22-bearing mouse. It is speculated that SJAMP enhances the immune function effectively.

Abdominal macrophage, which has phagocytosis, antigen presentation, immune regulation and anti-tumor function, plays an important role in anti-tumor immune response. Activated macrophages kill tumor cells by secreting cytotoxic factors such as TNF-α and NO indirectly. Compared to model group, abdominal macrophage phagocytosis was improved in SJAMP intervention groups, which suggested that abdominal macrophages had been activated. SJMAP enhances abdominal macrophage function likely by increasing Cytokines secretion and promoting macrophage cell surface receptor activation.

An important characteristic of activated lymphocyte is proliferation when stimulated by antigen or mitogen [15]. Lymphocyte proliferation experiment is a reliable indicator to evaluate this function [16]. The proliferation ability of spleen lymph cell in SJAMP groups is higher than in model group, indicating that there is a promotion of proliferation in SJAMP groups. The possible mechanism is SJAMP could improve lymphocyte activate ability.

TNF-α affects the growth, differentiation, and survival of all cells. Besides hemorrhagic necrosis can be induced by TNF-α in tumor cells, growth inhibition and cytotoxicity can also be mediated by TNF-α in tumor cells, as well as in virus-infected cells. In addition, with enhancement of macrophage, T cells and NK cells, TNF-α plays an indirect anti-tumor effect. But more and more studies have found excess TNF-α cause cachexia which promotes tumor progression[17,18]. TNF-α induces cell death in a dose-dependent manner[19]. A previous report showed that HCC in mice led an increase in serum TNF-α [20]. In the present experiment, there is a marked decrease in serum TNF-α level in 5-FU group and SJAMP groups(P<0.05). Regulating TNF-α secretion in the best level may be one of the anti-tumor mechanisms of SJAMP.

In conclusion, the anti-tumor effect of SJAMP is not as significant as 5-FU. Yet SJAMP with fewer side effects plays an important role in enhancing immune function which has attracted more and more attention in inhibiting tumor growth and preventing tumor metastasis and recrudesce. SJAMP is expected to be the important natural anti-tumor drug. In present study tumor growth was significantly inhibited and immune function was significantly improved. The results provide an experimental and theoretical basis for the study of the anti-tumor effects of SJAMP. SJAMP may be considered used with chemotherapeutics, to reduce the damage of chemotherapeutics to the immune system and improve the anti-tumor effect. This will provide new chemotherapy for the treatment of tumors.

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刺参酸性粘多糖对 H22 肝癌小鼠免疫功能的影响 *

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摘要 目的: 刺参酸性粘多糖作为一种天然生物活性物质,具有较强抗肿瘤作用。本研究观察刺参酸性粘多糖对荷瘤小鼠免疫功能的影响,以探讨刺参酸性粘多糖的抗肿瘤作用机制。**方法:** 皮下接种 H22 小鼠肝癌细胞,建立移植瘤小鼠模型。将 50 只荷瘤小鼠随机分为五组(阴性对照组、氟尿嘧啶组、SJAMP 低剂量组、SJAMP 中剂量组、SJAMP 高剂量组),腹腔注射不同剂量刺参酸性粘多糖,每日一次,连续 12 天。眼球摘除取血后颈椎脱臼处死小鼠,计算抑瘤率和脏器指数,中性红法测定小鼠腹腔巨噬细胞吞噬功能,CCK-8 法测定小鼠脾淋巴细胞增殖能力,ELISA 法测定小鼠血清 TNF-α 水平。**结果:** SJAMP 能够明显抑制肿瘤生长(P<0.05);与 5-FU 组相比,SJAMP 干预组脾指数和胸腺指数明显升高(P<0.05),腹腔巨噬细胞吞噬能力和脾脏淋巴细胞增殖功能显著提高(P<0.05),TNF-α 的血清含量显著减少(P<0.05)。**结论:** 刺参酸性粘多糖通过促进免疫器官生长,增强机体的免疫功能,抑制小鼠 H22 肝癌生长。这为 SJAMP 的抗肿瘤作用研究提供了试验依据和理论基础。

关键词: 刺参酸性粘多糖;H22;免疫

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