The Intervention Effect of SJAMP on Hepatocellular Carcinoma Rat Induced by Diethylnitrosamine and Impact on Immune Function*

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ABSTRACT Objective: To establish animal models of Hepatocellular Carcinoma in rats and to investigate the intervention effect of Stichopus japonicus acid mucopolysaccharide (SJAMP) on Hepatocellular Carcinoma and immune function. Methods: 50 male Wistar rats were randomly divided into five groups: normal control group, model group and SJAMP intervention groups (group A B C). The model group and SJAMP intervention groups were fed with 0.2 % DEN saline solution up to the 16th weeks to induce hepatocelluar carcinoma, meanwhile they were intervened with different doses of SJAMP during the entire carcinogenesis process, then all the rats were killed. Blood samples were drawn from the abdominal aorta, the spleen and thymus were removed under sterile conditions, and the spleen index and thymus index were calculated. The number of the nodules ≥ 3mm and ≥ 5mm, the size of the largest nodule was compared between groups. The tumor inhibitory rate was calculated. Adherent purified Mφ , phagocytosing neutral red method was used to detect macrophage phagocytosis and spleen macrophage killing capability was assayed by MTT method. Results: The number of the nodules ≥ 3mm and ≥ 5mm and the mean volume of the largest nodule of SJAMP intervention groups was significantly less than that in model group ($P<0.05$), and the tumor inhibition of SJAMP was significantly higher than model group; Compared with model group, the spleen indices and thymus indices of SJAMP intervention groups were increased significantly ($P<0.05$); The phagocytosis and killing capability of Mφ of SJAMP intervention groups were all higher than those in model group($P<0.05$). Conclusion: SJAMP has significantly inhibition on the occurrence of liver cancer in rats and the mechanism which may be stimulate the immune organ tissue enhance the body's cell-mediated immunity ability.

Key words: Diethylnitrosamine; Stichopus japonicus acid mucopolysaccharide; Macrophage; Liver cancer; Immune function

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Introduction

Primary liver cancer (primary hepatic carcinoma) is one of the most common malignancy. The incidence of liver cancer in China accounts for 40 % of the world each year, but 5-year survival rate of liver cancer is only 13 %[1]. Liver neoplasms have the lowest survival rate and has become the second cause of death in all the other cancers in China [2]. Stichopus japonicus acid mucopolysaccharide(SJAMP), one kind of polysaccharides, extracted from sea cucumber body wall. Present study shows SJAMP has broad-spectrum anti-tumor effect and inhibition on a variety of solid tumors and tumor cells[3,4]. It can be used to improve immune dysfunction induced by anti-cancer drug and tumor-bearing animals [5-7], or as inhibition of angiogenesis induced by transplanted tumor [6,8]. However, whether there is significant inhibition of SJAMP on primary liver cancer has not yet been confirmed. So this study used diethylnitrosamine to establish liver cancer animal model and intervene with different doses of SJAMP during the whole process of induction of liver cancer, to investigate SJAMP inhibition on primary liver cancer and its impact on immune function in rat.

1 Materials and methods

1.1 Materials

50 SPF male Wistar rats,130-150 g, purchased from Shandong Lukang Pharmaceutical Co., Ltd. (quality certification number: SLXK Lu 20,080,002); SJAMP, Ocean University of China School of Food Science and Engineering extract; N-nitrosodiethylamine (alias diethylnitrosamine), Sigma-Aldrich Corporation; Human hepatoma cell line HepG2, purchased from Chinese Academy of Sciences Shanghai Cell Bank; RPMI-1640 medium and D-Hank's, the United States Hyclone Company; 0.25 % Trypsin-EDTA digestion; Tetrazolium salt (MTT), Sigma-Aldrich Corporation; Neutral red, Solarbio company.

1.2 Apparatus and equipment

Clean Animal housing; FA1004N electronic balance, Adrian Shanghai Precision Scientific Instrument Co., Ltd.; Rayto microplate reader (RT-6000), Shenzhen Leidu Life Sciences, Inc.; MCO-20AIC 37 ℃ CO_2 incubator, the Japanese Sanyo company; DT5-1 centrifuges, centrifuge Beijing Times North Lee Co.; XDS-1B inverted biological microscope, Chongqing Optical Instrument Co., Ltd.; Thermo Scientific Forma 702 ultra-low temperature refrigerator, USA Thermo Fisher Scientific Co., Ltd.

1.3 Method

1.3.1 Animal Model After one week feeding adaptation, the

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rats were randomly divided into five groups, namely normal control group, model group and SJAMP intervention groups (A B C). The rats were fed with standard laboratory diet and water. In addition to the normal control group, model group and SJAMP intervention groups received 0.2 % concentration DEN saline solution ig (10 μg/g) for 16weeks, administered orally 5 days per week, 2 days off, continuing to the 16th week [9], meanwhile SJAMP intervention groups received SJAMP ig (respectively 0.175μg/g, 0.35μg/g,0.7μg/g) throughout the whole process of inducing liver tumors. Until the 16th week, all the rats were anesthetized with intra-peritoneal injection with 10% chloralhydrate (0.3 mL/100g). Blood was drawn from the abdominal aortic, the spleen and thymus were removed and weighed under sterile conditions. The tumor was took off, liver nodules sizes were measured with a vernier caliper, the number of nodules whose maximum >3mm and >5mm was counted, and the longest and shortest diameter of the largest tumor nodule were measured, inhibition rate was calculated according to the formula[10]:

Tumor volume (mm3) = π / 6 × a × b^2 (a is path for the long, b is the short diameter)

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

1.3.2 Preparation of spleen cell suspension Wash the spleen with D-Hank's solution 2 times, take 0.5 cm^3 spleen with sterilized scissors, the spleen was cut again and again, up until the paste appears, get sterilized 200-mesh nylon net and a small beaker, dumped the paste in the nylon net, grinding the paste gently while rinsing with D-Hank's solution, prepared cell suspension. The cell suspension was removed into centrifuge tube, 5 mL Tris-NH_4Cl was added to break red blood cells, stand for 5 minutes, then 1000r/min centrifuged for 15 minutes. Remove the supernatant, add RPMI1640 medium containing 10 % fetal calf serum, wind and percussion with a straw, added into the culture bottles at 37 ℃ 5 % CO_2 humidified incubator, after 2 to 3 hours, suck out the culture medium with a straw, and washed twice with D-Hank's solution, suck out the fluid, then the vast majority of adherent cells is macrophages, and non-adherent cells is mainly spleen mixed lymphocyte.

1.3.3 Thymus index, spleen index determination Spleen index = spleen wet weight (mg) / body weight (g)Thymus index = thymus wet weight (mg) / body weight (g).

1.3.4 Detect macrophage phagocytosis phagocytosing neutral red method [11] Digest the adherent macrophages with 0.25 % trypsin digestion prepare into a single-cell suspension, each sample was inoculated into three holes (1× 10^6/mL) in 96-well plates, cultured for 48 hours to 96 plate, then suck out 100 μL culture medium each well, and add 0.1% neutral red solution 100 μL, continue to incubate in 37 ℃ 5 % CO_2 humidified incubator for 30 minutes. Then suck out the supernatant, washed each well three times with PBS, and each well added 100 μL lysis buffer (0.1mol/L acetic acid: ethanol = 1:1), then put at 4 ℃ refrigerator overnight, determinated the OD value at 492nm with enzyme-linked immunosorbent tester.

1.3.5 Determination of macrophage killing function[12] Macrophage cell suspension prepared as above, adjusting the cell concentration (1× 10^6/mL), each sample set 3 holes in 96-well plate, take the macrophages as effector cells and HepG2 cells as target cells, collect the HepG2 cells in the logarithmic phase, adjust the cell concentration (5× 10^4/mL), add their suspension 100 μL respectively according to the ratio of 20:1. After 48 hours later, each well add 5 mg / mL MTT 20 μL, continue to incubate in 37 ℃ 5 % CO_2 humidified incubator for 4 hours, then centrifuge for 15 minutes at 1500r/min, discard supernatant, each well add DMSO150μL, after 15-minute oscillation measure the A value at 570 nm.

Macrophage killing activity(%)=[1-(OD value of expereimental hole-OD value of simple effect hole)/OD value of target cells hole]× 100%.

1.3.6 Data Processing The data using SPSS16.0 for One-Way ANOVA statistical analysis, the results show with the mean ± standard deviation, mean pairwise comparison with LSD-t test among groups.

2 Results

2.1 Growth conditions of rats in the feeding process

The growth of normal rats in good condition, body weight increased rapidly, gloss coat, normal urine. But for the first 8 week, the rats of model group and SJAMP intervention groups began to appear different levels of apathetic, dull coat, gloss loss, loss of appetite, reduced activity, slow growth, weight loss, static-like "hair up" phenomenon. The growth state of rats of group B and C were significantly better than the model group and group A. As shown in Table 1 and Table 2, the mortality rate of model group was significantly higher than SJAMP intervention groups, and the number of the nodules ≥ 3mm and ≥ 5mm of model group were significantly more than SJAMP intervention groups, and SJAMP had significant inhibitory effect.

2.2 Thymus index, spleen index

The thymus index of model group was significantly lower than control group($P<0.05$), but the thymus index of SJAMP intervention groups was higher than model group and showed a clear dose - response relationship (Table 3). The spleen index of model group and SJAMP intervention groups were significantly higher than the normal control group ($P<0.05$), and increased significantly due to SJAMP intervention ($P<0.05$), which showed that when the body was attacked by poison from outside, it could mobilize its own immune organs to defense against external toxins, and the effect could be promoted with SJAMP intervention.

Table 1 The effect of SJAMP on mortality of diethylnitrosamine-induced liver cancer in rat

Groups	n1	n2	n3	Mortality Rate
Control Group	10	10	0	0
Model Group	10	7	3	30%
Group A	10	9	1	10%
Group B	10	9	1	10%
Group C	10	10	0	0

Note: n_1 represents the number of animals in the beginning of the experiment; n_2 represents the number of animals in the end of the experiment; n_3 represents the number of cases of death during the experiment.

Table 2 The effect of inhibition of SJAMP on diethylnitrosamine-induced liver cancer in rat($\bar{x}\pm s$)

Group	n	The number of the nodules ≥ 3mm	The number of the nodules ≥ 5mm	Mean of the size of the largest nodule(mm^3)	Tumor inhibition (%)
Control Group	10	-	-	-	-
Model Group	10	46.57± 15.28	6.71± 1.86	198.84± 64.80	-
Group A	10	40.71± 16.29	3.89± 1.14	115.70± 26.61[a]	41.8
Group B	10	31.57± 9.42	3.28± 1.12	89.51± 23.97[a]	55.0
Group C	10	26.14± 7.14	2.57± 1.12	66.37± 18.00[ab]	66.6

Note: a: P<0.01, vs model group; b:P<0.01, vs group A.

Table 3 The impact of SJAMP on thymus index and spleen index of induced liver cancer in rats($\bar{x}\pm s$)

Group	N	Thymus index	Spleen index
Control Group	10	0.965± 0.159	1.165± 0.226
Model Group	7	0.571± 0.167[a]	3.364± 0.770[a]
Group A	9	0.706± 0.199[a]	3.491± 0.991[a]
Group B	9	0.928± 0.225[bc]	4.418± 0.601[ab]
Group C	10	0.994± 0.173[bc]	5.569± 0.969[abd]

Note: a:P<0.05,vs control group; b:P<0.05, vs model group; c:P<0.05, vs group A; d:P<0.01, vs group B.

2.3 Splenic macrophage phagocytosis of neutral red

Compared with that in the normal control group, phagocytosis of macrophages of model group and SJAMP intervention groups increased significantly (P<0.01), while SJAMP intervention groups showing greater macrophage phagocytic capacity (P<0.01), and the intervention dose and phagocytic activity showed a dose-response relationship, SJAMP play a role in the activation of splenic macrophages, which can enhance splenic macrophage phagocytosis (Table 4).

2.4 Comparison of Splenic macrophage killing function

Compared with that in the normal control group, the ability of killing tumor cells of macrophages in model group was significantly lower (P<0.01), which indicated that the liver cancer had inhibition to tumor-killing ability of macrophages; But SJAMP intervention will significantly increased cytotoxic activity, and there was significant difference between group C and model group (P<0.05). Compared with model group, cytotoxic activity in group A and B has a increase trend, but no significant difference (P>0.05), which may be related with small sample size (such as shown in Table 4).

Table 4 The impact of SJAMP on macrophage phagocytosis of neutral red and killing function($\bar{x}\pm s$)

Group	N	Phagocytosis(OD value)	Cytotoxic activity(%)
Control Group	10	0.078± 0.003	55.25± 10.33
Model Group	7	0.122± 0.012[a]	39.73± 5.19[a]
Group A	9	0.125± 0.008[a]	43.20± 8.54[a]
Group B	9	0.139± 0.015[ab]	47.70± 4.93
Group C	10	0.144± 0.011[ab]	57.12± 9.23[bcd]

Note: a: P<0.01, vs control group; b: P<0.01, vs model group; c: P<0.01, vs group A; d:P<0.05, vs group B.

3 Discussion

As a common chemical carcinogen, diethylnitrosamine has a clear affinity for the liver tissue, prolonged exposure to this toxic substance causes changes in liver, followed by nodular hyperplasia of liver cells and cirrhosis, and finally lead to liver cancer. For easy operation, high rate of induced cancer, typical cirrhosis during the induction of carcinogenesis process which was very similar compared to human liver cancer, and specific carcinogenicity, the significant proportion of tumor induced is hepatocellular carcinoma, accounting for 74.4 %[13], is an ideal model of human liver cancer, that has been widely used in liver cancer prevention and treatment research. Tumor development is closely related with the state of immune function. When the host immune function is suppressed, the tumor incidence increased. But when tumor growth in progressive, the tumor in patients with suppressed immune function, the interaction between the two, these two factors of growth and decline play an important role of tumor development and prognosis [14]. When the tumor occurs, on the one hand, by reducing their tumor immunogenicity, immune tolerance can occur with immune escape, induction of immune suppressor cells and immunosuppressive factors undermine the body's immune function, resulting in immune organ atrophy and dysfunction; On the other hand, a variety of immune effect mechanism of anti-tumor effect also occur in the body. Anti-tumor mechanism of the body included cell-mediated immunity and humoral immunity. In general, cell-mediated immunity plays a major role in the anti-tumor immune function. Generalized cell-mediated immunity also including innate immune cells, such as macrophages, natural killer cells, neutrophils, etc., can quickly response to the pathogen of the body, play the role of non-specific anti-inflammatory effect, and it can also remove the body injury, aging or distort cells, and participate in the adaptive immune response[15]. Thymus provides a place for T cell differentiation, development and maturation, play a regulatory role in the peripheral immune organs and immune cells [18]. The spleen is the major immune organs in animals, containing a large number of lymphocytes, macrophages, NK cells and K cells, with specific and non-specific systemic immune function [16]. Spleen index and thymus index can reflect the development of immune organs and immune cell function status. This study showed that SJAMP could stimulate and promote the growth of spleen and thymus. Increased spleen index and thymus index of cancer in rats induced, which suggested SJAMP could effectively prevent the immune dysfunction caused by immune organ atrophy and dysfunction, maintain and enhance the normal immunity of the body.

With phagocytosis, antigen presentation, immune regulation and anti-tumor cell functions, splenic macrophages is an important natural component of the immune response and plays an important role in anti-tumor immune response, and together with macrophages cells in the other tissues and organs to constitute the first line of defense to withstand external invasion, to clear pathogens, immune complexes and other foreign matter in the blood. When stimulated by foreign antigens such as pathogenic microorganisms or cytokines, the surface receptors are activated, the function can be significantly enhanced. It was found that compared to normal control group, splenic macrophage phagocytosis of neutral red were improved in model group and SJAMP intervention groups, which suggested that splenic macrophages had been activated maybe due to exposure to exogenous toxic substances. SJAMP intervention could further enhance their phagocytosis. SJMAP enhanced splenic macrophage function likely by promoting macrophage cell surface receptor activation. Tumor cell-killing function of macrophages in model group was below the normal control group, indicating that there was inhibition of macrophages in model group. The possible mechanism including poor lysosome fusion with phagocytic, reduced ability to commission antigen, Fc receptor closed or destruction[17], but with SJAMP intervention can be effective against exogenous toxins and inhibition of macrophages.

In summary, SJAMP can not completely block occurrence and evolution of liver cancer induced by its diethylnitrosamine which is recognized as a strong carcinogen, but this reseach showed SJAMP can inhibit liver tumor growth, protect the immune organs and tissue, promote proliferation of thymus and spleen of rats tumor-induced, enhance phagocytosis and killing ability of splenic macrophages to tumor cells, therefore, it is still a high valuable substance to prevent the development of liver cancer.

References

[1] Jiang WQ, Zhang XS, Zhu XF, et al. Tumor biological therapeutics [M]. Guangzhou: Guangdong Science and Technology Publishing House, 2006:609-611

[2] Stefaniuk P, Cianeiara J, Wiercinska DA. Present and future possibilities for early diagnosis of hepatecellular carcinoma [J]. World J Gastroenteral, 2010,16(4):418-424

[3] Hu RJ, Yu SP, Jiang H, et al. The inhibitory effect of SJAMP combined with cortisone on murine solid tumors [J]. Chinese Journal of Cancer, 1997,16(6):422-424

[4] Li HF, Chen Y, Yang MS, et al. Research of echinoderms natural products[J]. Chin J Mar Drugs, 2008, 27(4):52-59

[5] Li WM, Wang SH, Liang XQ, et al. The effects of acidic mucopolysaccharide from Stichopus Japonicus Selenka (SJAMP) on the immune function of mouse [J]. Chinese Journal of Clinical Oncology, 1985, 12:118-121

[6] Chen ZQ, Wei S, Jiao Y, et al. Effect of acidic polysaccharide from holothuria leucospilota on immunologic function of mice [J]. Tianjin Medical Journal, 1987, (5):278-280

[7] Sun L, Xu YH, Xu HL. Research of Stichopus japonicus acid mucopolysaccharide enhance cellular immune fuction [J]. Progress In Biochemistry and Biophysics, 1991,18:394-395

[8] Liang YJ, Wei SJ. Observe The effect of Stichopus japonicus acid mu-

copolysaccharide on histopathology and utrastructure of mice[J]. Chinese Journal of Integrated Traditional and Western Medicine, 1988: 61-62

[9] Cheng YN, Wang BF, Liu ZG, et al. Immunoregulatory effects of saikosaponin-d in rats with experimental hepatocarcinoma [J]. World Chinese Journal Digestology, 2009, 17(24):2451-2455

[10] Zhang MG, Wu MC, Chen H, et al. A study of effects of TNP-470 on the hepatocarcinoma Induced by DENA in rat[J]. Journal of Oncology, 2002,8(1):43-45

[11] Wang PS, Liu XL, Liu DY, et al. Inhibitory effect of the polysaccharides from codium fragile on carcinoma Hca-F bearing mice [J]. Chin J Mar Drugs, 2010, 29(5):40-43

[12] Guo LY, Wang L, Zou XY, et al. Antitumor and immuno modulating effects of Apostichopus japonicus polysaccharides (AJPS)in tumor-bearing mice[J]. Chinese Journal of Microecology, 2009,21(9):806-808

[13] Zhou ZZ, Zhang ZJ, Qian ZY, et al. Study on morphologic features during DEN-induced hepatocellular carcinomas in rats [J]. Academic Journal of Kunming Medical College, 2005, (3):19

[14] Xiang BD, Zhao YN, Yuan WP. The clinical characteristics of 143 cases of patients with primary liver cancer under the age of 30[J]. Cancer Research and Clinic, 2009,21(9):626-628

[15] Chen WF, Medical Immunology [M]. Beijing: People's Health Publishing House, 2001:223-224

[16] Jin BQ. Medical Immunology [M]. Beijing: People's Health Publishing House, 2008:16-18

[17] Qiu PC, Chen JS. Effects of splenectomy on anti tumor immunity during the induction of hepatocellular carcinoma in rats [J]. Chinese Journal of Bases and Clinics in General Surgery, 2000,7(4):224-225

[18] Jia QB, Liang XY, Wu GL, et al. Clinical significance of TNF-α,IL-2, sIL-2R in serum with primary hepatic carcinoma and liver cir- rhosis [J]. Chinese Journal of Gastroenterology and Hepatology, 2002,11(3): 243-248

刺参酸性粘多糖对诱发性肝癌大鼠的干预作用及免疫功能的影响*

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摘要 **目的**:通过建立诱发性大鼠肝癌动物模型,研究用刺参酸性粘多糖(SJAMP)对大鼠诱发性肝癌的干预作用及免疫功能的影响。**方法**:将雄性 Wistar 大鼠 50 只随机均分为 5 个组,正常对照组、模型组和 3 个 SJAMP 干预组(A 组,B 组和 C 组),模型组和 SJAMP 干预组灌胃 0.2%DEN 生理盐水溶液以诱发肝癌,同时 SJAMP 干预组按照不同剂量 (0.175μg/g, 0.35μg/g, 0.7μg/g) 给予 SJAMP,至 16 周处死大鼠,取血,无菌取脾、胸腺,计算脾指数、胸腺指数。比较各组 >3mm 和 >5mm 的结节数以及最大结节的体积,计算肿瘤抑制率。贴壁法纯化巨噬细胞,用中性红吞噬实验检测巨噬细胞吞噬功能,MTT 法检测巨噬细胞杀伤功能。**结果**:SJAMP 干预组 >3mm 和 >5mm 的结节数明显少于模型组,最大结节的平均体积明显小于模型组(P<0.05);与模型组相比,SJAMP 干预组脾指数和胸腺指数明显升高(P<0.05),SJAMP 干预组巨噬细胞吞噬能力和杀伤功能显著提高(P<0.05)。**结论**:刺参酸性粘多糖对大鼠诱发性肝癌有明显的抑制作用,其机制可能是通过刺激免疫器官生长,增强机体的细胞免疫能力来实现的。

关键词:二乙基亚硝胺;刺参酸性粘多糖;肝癌;巨噬细胞;免疫功能

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