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THE PREVENTIVE EFFECT AND ENHANCE IMMUNITY FUNCTION OF BU-ZHONG-YI-QI WAN ON S180 TUMOR MICE.

Fang Hu[1,2], Xiao-ya Liu[1], Xin-yue Chen[1], Can Li[1], Rui-juan Zhu[1], Huan Han[1], Ying-dong Li[3], Shi-lan Feng[*1]

[1] School of Pharmacy, Lanzhou University, 199 Donggang west Road, Lanzhou 730000, China. [2] The First Hospital of Tianshui, 105 Jianshe Road Qinzhou District, Tianshui 741000, China. [3]Department of Pharmacy, Gansu University of traditional Chinese medicine, 35 Dingxi east Road, Lanzhou 730000, China

***Corresponding author: E-mail:** fengshl@lzu.edu.cn

Abstract

Background: To evaluate the preventive effect and enhance immunity functions of Bu-zhong-yi-qi wan *in vivo*.
Materials and Methods: S180 tumor mice model was established by subcutaneous injection a dose of 0.2 ml (1×10^7/ml) at the right oxter. The inhibitory rates, spleen indexes and thymus indexes were calculated. Splenic lymphocyte proliferative activity assay and phagocytosis activity of peritoneal macrophages were done. Interferon (IFN-γ), interleukin (IL-2) and tumor necrotic factor (TNF-α) in serum were detected.
Results: In the S180 tumor-bearing mice, Bu-zhong-yi-qi wan with medium-dose (975 mg/kg, 100 mg/l) had potent preventive effect and anti-tumor effect, macrophage phagocytosis and Con A-stimulated splenocyte proliferation were increased as compared with model control treatment. Bu-zhong-yi-qi wan could take part in the immune response by promoting the proliferation and differentiation of T-cells, increasing the activity of the macrophages, inducing the generation of cell factor IL-2, TNF-α, IFN-γ.
Conclusion: It proved that Bu-zhong-yi-qi wan of medium-dose had great preventive effect and could enhance immunity function.

Key words: Bu-zhong-yi-qi wan, S180 tumor-bearing mice, preventive, immunity

Introduction

Malignant tumor is one of the major causes of death in the world despite newly developed tools and miracle drugs for treatment (Chen, L. et al. 2011) Radiotherapy and chemical therapy are used to treat tumor in clinical treatment, but acute side effects of radiotherapy can occasionally be life threatening (Ye, M., et al. 2013). Chemical therapy is one of the most important clinical methods to treat carcinoma (Zhao, et al. 2007), the frequently used chemotherapy drugs can kill tumor cells and simultaneously have a certain degree of killing effect on normal cells (Huang, D.N., et al. 2004). In addition, serious side effects such as loss of appetite and weight, body weakness will be caused (Miko, I., et al. 2005), and the main reason of death caused by malignant tumor is that immune systems were damaged in tumor treatment (Chen, Y.T., et al. 2012). In recent years, increasing the human body's immunity in defending against tumors has become a hot research area (Hussaina, M., et al. 2012; Wang, M., et al., 2012) Therefore, it is very important to investigate novel anti-tumor substances with preventive effect and improving immunity potential.

T cells is a major component of lymphatic cells; it has a variety of biological functions, such as killing target cells directly, supporting or inhibiting B cells to produce antibodies, promoting mitogen responses and producing cytokines and so on. It is the body to resist disease, tumor and form the heroic fighter. Interleukin 2 (IL-2), by virtue of its *in vivo* action in autocrine and paracrine manners, participates in the induction of both acquired and innate immunity. IL-2 has a stimulatory effect on activated T cells. Among cytokines applied as immunomodulators, IL-2 is the most commonly used for antitumor therapy (Jon M. Wigginton, et al. 2015), which indicates IL-2 is indirectly related to tumor. So in the experiment of anti-tumor, the determination of IL-2 is very important. TNF-α is a potential cytokine which can directly inhibit and kill tumor cells, and it is directly involved in apoptosis of tumor cells (Chen, Y.T., et al. 2012). IFN-γ promotes T cell differentiation and plays an important role in specific immunological reactions to tumor cells growth and promotes innate and adaptive immune responses (Yang, L., et al. 2003).

Bu-zhong-yi-qi-Wan (BW) composed of eight commonly used Chinese drugs, Radix Astragali (*Astragalus membranaceus* (Fisch.) Bge), Radix Glycyrrhizae (*Glycyrrhiza uralensis* Fisch.), Radix Codonopsis (*Codonopsis pilosula* (Franch.) Nannf.), Pericarpium Citri Reticulatae (*Citrus reticulate* Blanco), Radix Angelicae (Angelica sinensis (Oliv.) Diels), Radix Bupleuri (Bupleurum chinense DC.), and Rhizoma Cimicifugae (Cimicifuga foetida L.), was originated from the famous Bu-zhong-yi-qi-Tang (BT). In traditional use, BW is mainly used for spleen-qi descending, visceroptosis with hyposplenic qi, uterine prolapse, and rectal prolapse due to chronic diarrhea (National Pharmacopoeia Committee. Pharmacopoeia of Peoples Republic of China, 2010) BW and BT are the same composition but different dosage form. In traditional Chinese medicine, different dosage forms have different function. There are many reports about BT; BT has been used in the treatment of patients with perennial allergic rhinitis (Yang, S.H., and Yu, C.L. 2008), can be used to treat the symptoms such as weakness caused by fatigue and weakness after illness (Zheng, X.F., et al. 2014), and can be used for the treatment of various immune-related diseases (Yao, H.T., et al. 2012), and so on. However, large dose, long processing time, inconvenience for carrying, difficulties in quality control are the familiar disadvantages of medicinal broth in the clinical application (Xue, R., et al. 2011). The pill is relatively peaceful, and it is more durable than decoction (Li X.L., et al. 2008). But there are few reports about BW's

pharmacological effects. Because of convenience for storage, transport, carrying and being taken as pills, BW has a large number of uses in clinical applications. So the study of BW is very necessary.

In the present study, we evaluated the effects of BW on S180 tumor mice models. We found that BW has the preventive effect and could improve immunity of tumor-bearing mice, and medium-dose of BW significantly inhibited tumor growth.

Materials and Methods

Equipment

BP 211D electronic analytical balance (Sartorius AG, Germany), BS 224S electronic balance (Sartorius AG, Germany), microplate reader, desktop electric centrifuge (80-2, Changzhou guohua instrument co., LTD), MIKRO 22 (Germany) were used for the pharmacodynamics study.

Materials

BW (lot no. 12J50), the condensed pills derived from the aqueous extract of a mixture of 8 medicinal herbs, was provided by Lanzhou Foci Pharmaceutical Co. Ltd. Cyclophosphamide for injection (0413002) was purchased from Shanxi Pude Biopharmaceutical Co. Ltd. (Shanxi, China). Mice interleukin-2 (IL-2) Elisa interferon-γ Elisa and tumor necrosis factor-α (TNF-α) Elisa kits, nylon net filters, mouse lymphocyte separation medium (Lot: 33R021307-1) were all purchased from Dakota for biological technology co. Ltd. (Shenzhen, China). Chicken red blood cells (CRBCs) used in experiment were purchased from Guangzhou Ruite biological technology co. Ltd.

Reagents

Sodium chloride injection (Batch No. 2012092705) was purchased from Gansu Fuzheng pharmaceutical co. Ltd. (Lanzhou, China). ConA (Sigma: C-2010). MTT was come from Beijing Biotopped science & Technology co. Ltd. DMSO (20091126) was purchased from the Hanlon BoHua (tianjin) pharmaceutical chemical co. Ltd. Nutrient broth (20120509) was purchased from Qingdao high-tech park haibo biological technology co. Ltd.

Animals

One hundred and sixteen Kunming mice (58 male and 58 female), with the body weight of 20 ± 2 g, were purchased from Experimental Animal Center of Lanzhou University and housed in pathogen-free conditions in accordance with the principles approved by animal ethical committee of Lanzhou University and allowed free access to food and water. The animals were kept at 23 ± 2 ℃, with the humidity of 55 ± 5%, and cultured in a 12 h:12 h light–dark cycle.

Study Procedures

We analyzed the main components in BW. The main compositions of BW were analyzed by HPLC-UV-ELSD, the samples were ultrasonically treated with methanol, and then was purified using Waters Oasis HLB solid-phase extraction (SPE) column with our previous method (Fang H., et al. 2015).

Preparation of S180 Tumor-Bearing Mice

The grafting of animal with S180 sarcoma was following the method described by Li Wen-ping with some modification (Li, W.P., et al. 2006) Six Kunming mice (three male and three female) were intraperitoneally injected with 1 ml (2×10^6/ml) cell suspension for tumor cell proliferation in vivo, respectively. After breeding for 7 d, ascites of the mice under good growth status were gathered and diluted with sterile saline to a concentration of 1×10^7/ml. The S180 tumor cells were inoculated into the 110 Kunming mice by subcutaneous injection at the right oxter at a dose of 0.2 ml per mouse. The animals were randomly divided into 11 groups, each consisting of 10 mice.

Prevention group (PG): 50 animals were randomly divided into the 1 blank control group, 1 model group and three experimental groups. Before inoculated the mice in three experimental groups were respectively administrated with BW of low, medium and high dose at 487.5, 975 and 1950 mg/kg once daily for continued 10 days; the same volume of vehicle was orally administered to the control mice and model mice. At day 11, mice in model control group and three experimental groups were inoculated, and then normal bred for ten days without any process.

Enhance immunity group (EG): Twenty-four hours after inoculation, 60 animals were randomly divided into the 1 blank control group, 1 model group, 1 positive control group and three experimental groups. The mice were administrated with BW of low, medium and high dose at 487.5, 975 and 1950 mg/kg once daily for continued 10 days, the same volume of vehicle was orally administered to the control mice and model mice (Liu, S., et al. 2006)

Evaluation of Preventive Effect and Enhance Immunity Function

Determination of Tumor Inhibition Rate and Immune Organ Indices

Mice were killed by cervical dislocation on 11th day after inoculation and spleens were removed and weighed in sterile condition.

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The tumors were separated and weighed, the inhibitory rates were calculated. Thymuses were taken and weighed, and organ indices were calculated.

$$\text{Inhibitory rate } (\%) = \frac{\text{Tumor weight of the mouse in model control group} - \text{Tumor weight of the mouse in treatment group}}{\text{Tumor weight of the mouse in model control group}} \times 100.$$

Preparation of Spleen Cell Suspension

Plug grinding spleen with a syringe, then filter to a petri dish with fine nylon net filters, which with 5 ml of spleen cell suspension in advance, then transfer to a 10 ml centrifuge tube, add in 200 µl medium, then centrifuge 20 min with 2800 rpm, take the middle tier to a 5 ml centrifuge tube, blending, 1500 rpm, centrifuge for 10 min, the cell pellets were suspended in 1 ml medium.

Splenic Lymphocyte Proliferative Activity Assay

The MTT test was used following the method described by Zhong et al with little modifications (Zhong, K., et al. 2010). The concentration of above prepared splenic lymphocyte solution was adjusted to 1×10^7 cell/ml. Then 100 µl splenic lymphocyte solution was placed in a 96-well flat-bottom plate. A 30 µg/ml of Con A as a T-cell mitogen (100 µl) was added to the cell suspension. The mixture was incubated at 37 ℃ with 5% CO_2 for 24 h, 20 µl 5 mg/ml MTT was added into each well and incubated for 4 h. Subsequently, the plates were centrifuged (1400×g, 5 min) and the untransformed MTT was removed by pipetting. DMSO (150 µl) solution (192 ml DMSO with 8 ml 1M HCl) was added to each well, and the plates were mixed. After the purple crystal completely dissolved, the absorbance at 570 nm was evaluated using the microplate reader. 100 µl samples were incubated without canavalin A (Con A) as controls and the other 100 µl were incubated with Con A in a humidified atmosphere with 5% CO2 for 24 h. OD values were measured as previously described.

The rate of cell proliferation (%) = [(T−C)/C] × 100%, where T is the OD value of the test wells and C is the OD value of control wells.

Phagocytosis Activity of Peritoneal Macrophages

Phagocytosis of peritoneal macrophages was detected as the method described by Yang (Yang, S.H., et al. 2008). All mice were injected with 1 ml Starch broth (2%, v/v, with normal saline) at day 9, 10, 11 after inoculation to active peritoneal macrophages. Peritoneal macrophages were prepared from peritoneal exudates of mice after stimulation by intraperitoneally injection of 1 ml 2% (v/v) CRBCs 20-30 min before experiment. After mice were euthanized by cervical dislocation, 1 ml of macrophage solution was collected, adjusted the concentration to 1×10^7 cell/ml, took a drop spread onto glass slides, airing in room temperature, the cells were fixed with 1:1 acetone-methanol for 10 min and stained with Giemsa dye for 5 min. The number of macrophage-ingesting CRBCs in at least 100 cells was calculated by direct visual enumeration on light microscopy.

The phagocytic rate (PR) was calculated as follows:

$$PR(\%) = \frac{\text{number of macrophage} - \text{ingesting CRBCs}}{\text{total number of macrophages}} \times 100$$

Measurement of IFN-γ, IL-2 and TNF-α

The blood was collected by eye enucleation and the serum was separated by centrifugation at 3000 rpm for 10 min at 4 ℃. Determinations of IFN-γ, IL-2 and TNF-α were performed in accordance with the kit instructions at room temperature.

Statistics Analysis

All data were expressed as mean ± SD. Statistical significance between groups was determined using t-test. The significance of difference was considered to be obvious as $p < 0.05$.

Results

The Main Components in BW

Using our previous method (Fang H., et al. 2015), ten major components: hesperidin, senkyunolide I, senkyunolide H, ligustilide, butylene phthalide, ononin, calycosin, formononetin, astragaloside I, astragaloside IV in BW were determined in BW, so the main compounds in BW are these three categories: flavonoids, saponins and volatile oils.

Organ Index and Tumor Inhibition Rate

Table 1 showed the organ indexes, inhibition rates of cyclophosphamide of BW at varied dose on the S180 tumor in vivo. BW with

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three doses all had antitumor effect. In experiment PG, tumor inhibition rate of BW at high-dose reached to 64.75%, in experiment EG, tumor inhibition rate of BW at medium-dose reached to 75.63%, which was higher than positive group.

Table 1: Effects on tumor inhibition rate and organ index of mice with S180 sarcoma (n=10, mean ± SD）

	Groups	Inhibitory rates (%)	Thymus index	Spleen index
	Control group		25.85±5.08	61.97±2.12
PG	Model group		15.06±3.79	53.43±5.41
	High-dose group	64.75	20.52±3.54*	57.72±5.01
	Medium-dose group	59.81	15.60±3.86	60.27±5.25*
	Low-dose group	48.57	17.05±3.95	45.47±1.96
	Controll group		34.34±6.16	67.58±7.16
EG	Model group		26.74±5.70	64.60±7.16
	Positive group	74.67	28.81±3.31	65.00±6.40
	High-dose group	49.44	22.75±6.11	55.51±4.89
	Medium-dose group	75.63	29.00±7.60*	54.05±5.16
	Low-dose group	43.41	25.62±7.60	53.66±4.16

Note: PG: Prevention group, EG: Enhance immunity group. Compared with the model group * p <0.05

Effects of BW on the Evaluation of Splenic Lymphocyte Proliferative Activity

Data in Figure 1 showed BW treatment in experiment PG had significant effects on the Con A-stimulated splenocyte proliferation as compared with model control treatment ($p < 0.05$). But in experiment EG only high-dose had significant effect on the Con A -stimulated splenocyte proliferation as compared with model control treatment ($p < 0.05$).

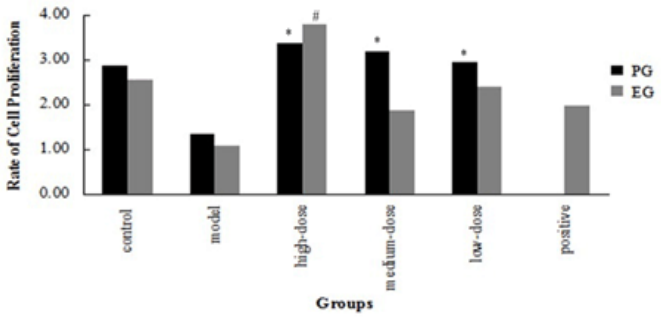

Figure 1: Effects of BW on the evaluation of splenic lymphocyte proliferative activity. *: p <0.05 compared with the model group in PG group; #: p <0.05 compared with the model group in EG group. PG, Prevention group; EG: Enhance immunity group.

Effects of BW on the Evaluation of Phagocytosis of Peritoneal Macrophages

The phagocytosis of peritoneal macrophages isolated from mice models was shown in Figure 2. As shown, the phagocytic activity of macrophages of treatment groups both in experiment PG and EG all enhanced as compared with that of model control group, and the maximum phagocytic rate was obtained (42.00%, 38%) in PG and EG. In the other hand, ANOVA showed the phagocytic rates.

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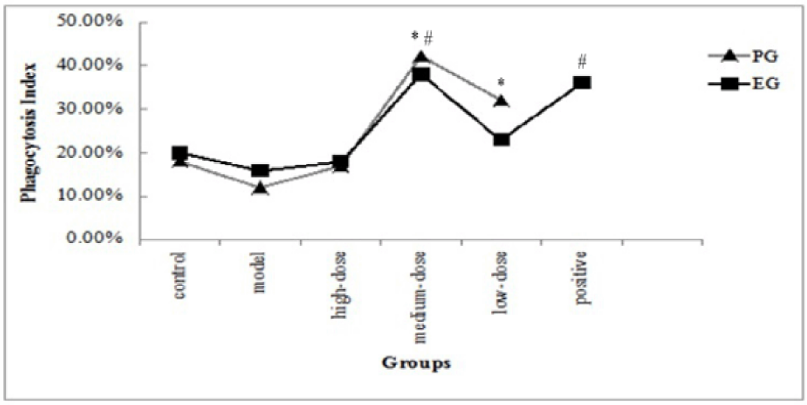

Figure 2: The phagocytosis indexes of BW in different groups. *: p <0.05 compared with the model group in PG group; #: p <0.05 compared with the model group in EG group. PG, Prevention group; EG: Enhance immunity group.

Measurement of IL-2, TNF-α and IFN-γ

Compared with the model group, IL-2, TNF-α and IFN-γ of mice in BW groups and positive control group all increased to varying degrees, and which were all close to those of mice in normal group ($p < 0.05$) except TNF-α both in high-dose BW groups and IL-2 of experiment EG in high-dose BW group (Table 2). The results showed that BW of medium-dose had remarkable antitumor activity, but BW in high-dose might have a opposite function.

Table 2: The content of IL-2, TNF-α and IFN-γ (n=10, mean ± SD).

	Groups	TNF-α (pg/ml)	IL-2 (pg/ml)	IFN-γ (pg/ml)
PG[1]	Control group	43.44±1.46	7.91±1.23	14.09±2.85
	Model group	41.45±1.26	5.16±1.73	11.93±2.17
	High-dose BW group	31.88±1.58	7.62±1.67*	12.72±3.25
	Medium-dose BW group	49.07±2.21*	5.86±2.55	13.25±1.98*
	Low-dose BW group	47.59±2.69*	7.87±1.14**	12.75±1.06
	Control group group	55.58±3.87	5.02±1.31	11.59±2.14
	Positive group	59.84±4.56**	4.17±1.44*	7.95±1.46**
EG[1]	Model group	49.55±4.15	3.70±0.81	6.88±1.75
	High-dose BW group	37.95±3.79	3.58±0.55	7.06±1.67
	Medium-dose BW group	55.98±4.23*	4.96±1.23*	7.19±1.19*
	Low-dose BW group	50.62±4.08	4.20±1.57*	6.90±0.81

Note: PG: Prevention group, EG: Enhance immunity group. Compared with the model group * p <0.05

Discussion

Thymus and spleen are important immune organs of animals. They achieve the purpose of tumor suppression through taking part in immune responses, regulating cellular immune and promoting tumor cells apoptosis (Miao J., et al. 2012). Compared with the model group, in experiment PG, the thymus indices of mice in varied dose BW group were elevated in different degrees; in experiment EG the thymus indices of mice in medium-dose group were elevated. It was worth noting that the thymus indices of mice in experiment PG were all elevated than model group, but in experiment EG only medium-dose group were elevated, which might indicate BW have a good prevention effect on S180 mice. Additionally, the spleen indices of mice only in experiment PG were significantly different from those in model group. The thymus indices and spleen indices of the mice in positive control group in experiment EG were reduced, indicating that cyclophosphamide caused a decline in immune function to a certain extent (Yang, Q., et al. 2007).

Conclusion

Effects of BW on immunomodulatory and preventive effect of tumor on mice models inoculated with S180 tumor cells were evaluated in this paper. The results showed that BW with medium-dose had potent immunomodulatory and preventive effect on tumor properties in mice models, including significantly enhanced on macrophage phagocytosis and Con A-stimulated splenocyte proliferation as compared with model control treatment, and BW with experimental doses had extreme antitumor effects, and the maximum inhibition rate reached to almost 75.63%. The results of organ indexes, inhibition rates of cyclophosphamide indicated the BW with three doses all had antitumor effect. The measurement results of IL-2, TNF-α and IFN-γ of mice showed BW of medium-dose had remarkable antitumor activity. Therefore, BW could be explored as a great and potential immunomodulator in the field of pharmaceutical and functional foods and further studies are needed to investigate the physiological and pharmacological properties of BW.

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