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Original Article

Beneficial effects of jiawei Baihe Dihuang Tang, a Chinese herbal decoction, in mouse hepatocellular carcinoma

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Abstract: An alternative treatment for hepatocellular carcinoma (HCC) which is a kind of common fatal human disease needs to be developed HCC becomes highly resistant to chemotherapeutic drugs during treatment. In this paper we aim to explore the effect of Jiawei Baihe Dihuang Tang (JBDT) on HCC via observation of index variation in mouse model. HCC mouse model were established using ICR mice. HCC mice were treated with Cyclophosphamide (CTX), JBDT with three different dose levels separately. Groups of ICR mice without any treatment and HCC mice without any drug were set as control. Mice were sacrificed after treatment with CTX or JBDT for 16 days. Tumor tissue, spleen and thymus were weighed and compared with control mice. Flow cytometry for cell cycle and immunohistochemistry for proliferating cell nuclear antigen (PCNA) were applied to evaluate the effects. Either CTX or JBDT of high dose level significantly lowered tumor tissue weight, reduce PCNA expression and block the tumor cell in G1 phase. Besides, CTX had some side effects during treatment, but JBDT enhanced immune function, protected spleen and thymus of HCC mice while CTX increased the amount of red blood cell and leukocyte and prevented leukocyte reduction in the process of treatment. Our results suggest JBDT can significantly inhibit tumor growth in a dose-dependent manner and avoid the side-effect of chemotherapy. JBDT is a potential drug of HCC treatment although the clinical application of JBDT still needs further exploration.

Keywords: Hepatocellular carcinoma, jiawei baihe dihuang tang, side effects, cyclophosphamide, mouse model

Introduction

Hepatocellular carcinoma (HCC) is one of the most common fatal human disease and highly resistant to chemotherapeutic drugs [1]. Cyclophosphamide (CTX) is the most conventional chemical used for HCC treatment. However, chemotherapy causes serious toxic effects [2]. Thus, there is an urgent need to develop novel treatment modalities.

Traditional herbal formulae have been clinically used for thousands of years in China. Nowadays, the use of traditional herbal formulae has provided us a prospective alternative in the treatment of HCC [3]. Baihe Dihuang Tang (BDT) is a renowned Chinese herbal formula and firstly described in "synopsis of the Golden Chamber" (Jinkui Yaolue) written by Zhang Zhong Jing in the early 3th century. JBDT is additionally made with astragalus in the basis of BDT. It is composed of three component herbs: astragalus, lilybulb (Bulbus Lilii) and rehmannia root (Radix Rehmanniae). Researchers found that astragalus had an inhibitory effect on airway inflammation through modulating the imbalanced relationship between Th1 and Th2 cytokines [4] and can enhance cellular and humoral immunity. Besides, astragalus facilitated removal of circulating immune complexes and induced IL-2 expression [5]. Dong et al. reported that Baihe (lilybulb) had inhibitory effects on tumor growth and metastasis of gastric cancer orthotopically transplanted in nude mice by down-regulating the expression of VEGF and p53 [6]. Qin et al. found that Xijiao Dihuang decoction could protect nerve cell by reducing the expression of caspase-3, TNF-alpha and IL-6, increasing the

expression of Bcl-2 after ICH in hemorrhagic brain damage [7]. Dihuang polysaccharide-B, which is the effective component of Dihuang, can repress tumor growth by affecting function of immune system [8]. However, what effects JBDT will produce on HCC and mechanism under this process are still unknown.

In the present study, we aimed to investigate the effects of JBDT on HCC in mice. JBDT of three different dose levels and CTX, which is a chemotherapy agent, were used to explore anti-tumor effects. Out results provide important parameters and phenotype for future clinical study.

Material and methods

Chemicals and reagents

CTX was purchased from Hengrui Medcine Ltd (Lianyungang, Jiangsu, China). 0.9% NaCl solution was supplied by Huayu Medcine Ltd (Wuxi, Jiangsu, China). PCNA primary antibody, SABC kit and DAB reagent for immunohistochemistry were obtained from Boster Company (Wuhan, China).

Preparation of JBDT

Bulbus Lilii (BL), Radix Rehmanniae (RR) and Astragalus (AG) were provided by pharmacy of Zhejiang Chinese Medical University. JBDT was formulated by mixing the three herbal in relative proportions according to a ratio of 2:1:2 (BL:RR:AG). After decoction, filtration and concentration, this extract was prepared as a 1 g/ml solution, which was aliquoted, sterilized and stored in the desiccator at 4°C until use.

Animal and tumor cell line

Half male and half female ICR mice weighting 18-24 g were obtained from the Laboratory Animal Center, Medical Academic Sciences, Shanghai, China. The animals were maintained in Clean Animal Center of Zhejiang Chinese Medical University. HCC cell line H22 was provided by Animal Center of Zhejiang Chinese Medical University.

HCC mouse model

Fresh tumor tissue of mouse was taken to prepare single-cell suspensions. The concentration of the suspension was adjusted to 6×10^6/ml using 0.9% NaCl. 0.2 ml suspension was inoculated into the left leg of ICR mouse to build HCC model.

Group and treatment

The animals were randomly assigned into groups of 10 individuals. 0.9% NaCl was given to animals in group 1 (ICR mice, negative control group) and group 2 (HCC mice, positive control group) at 20 ml/kg/day. Animals in group 3 were administered with chemotherapy compounds (CTX, 100 mg/kg/day) and 0.9% NaCl (20 ml/kg/day). Animals in groups 4, 5, and 6 received intragastric doses of JBDT extract at 7.5 g, 15 g and 30 g/kg/day, respectively.

Mice were weighted and draw blood from vein of post-eyeball before transplanted with HCC tumor cell and at 16^{th} day of HCC model building. Red blood cell (RBC) and leukocyte were counting after blood collecting. Mice were sacrificed by taking off cervical vertebra. Tumor tissue, spleen and thymus were dissected and weighed separately.

Immunohistochemistry

Paraffin embedded sections were routinely processed. Antibody dilutions were as follows: PCNA-1:100. All following procedures were done according to standard protocols with Dako EnVision Systems. The results of PCNA protein staining cell were classified as follows: negative-no staining seen in HCC tumor cells, positive--weak or strong brown granules staining seen in HCC tumor cells.

Five horizons were random chose for every section. PCNA negative cells per 1000 cells were defined as PCNA labeling index (LI). Sections were classified as follows: weak positive PCNA expression (+-)-PCNA LI is from 40% to 60%, positive PCNA expression (+)-PCNA LI is from 60% to 80%, strong positive PCNA expression (++)-PCNA LI is above 80%.

Flow cytometry

Single tumor cell suspension were prepared at a concentration of 1×10^6/ml and fixed with 75% cold ethanol. Cells were Washed with pre-cold PBS and stained using 1 ml PI staining buffer at 4°C for 20-30 min (200 ml buffer includes: 129.6 ml 0.9% NaCl, 10 mg PI, 2 mg RNase,

Table 1. Tumor Inhibitory rate under treatment in each group ($\bar{x}$±s)

Group	Quantity (n)	Tumor weight (g)	Inhibitory rate (%)[a]
Healthy control	10	/	/
HCC mice group	10	2.35±0.51	/
CTX group	10	1.34±0.51	42.97
Low dose JBDT	10	2.10±0.23	10.64
Middle dose JBDT	10	1.86±1.02	20.85
High dose JBDT	10	1.54±1.30	34.47

a: inhibitory rate = (1-average weight of tumor in drug treatment group/average weight of tumor in HCC model group) ×100%.

Table 2. PCNA expression after treatment in each group

Group	Quantity (n)	PCNA negative cell amount (%)
Healthy control	10	/
HCC mice group	10	66.0±10.53
CTX group	10	$30.63±13.56^{\Delta\Delta}$
Low dose JBDT	10	$65.77±12.12^{**}$
Middle dose JBDT	10	$45.61±11.73^{\Delta,**}$
High dose JBDT	10	$38.51±9.20^{\Delta\Delta}$

$^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$,comparison against HCC mice group, two-tailed *t* test; $^{*}P<0.05$, $^{**}P<0.01$, comparison against CTX group, two-tailed *t* test.

0.5 ml 1% Triton X-100, 200 mg Sodium Citrate, adding water to 200 ml.), then discarded supernatant after centrifugation and resuspended pellet with PBS and loading samples on instrument. Software MultiCycle for windows 4.0 was used for data analysis.

Statistical analysis

All data were analyzed using software SPSS 11.0. The present data are expressed as mean ± SD. For statistical comparison, Student's *t* test and ANOVA were used. *P* values less than 0.05 were deemed to indicate statistical differences (*$P<0.05$). *P* values less than 0.01 were deemed to indicate statistical significant differences (**$P<0.01$).

Results

JBDT can inhibit tumor growth in a dose-dependent way

Expansion of tumor tissue is an important characterization of cancer. Here we applied different dose level of JBDT and observed the efficacy of JBDT on HCC mice. 60 mice were randomly divided into 6 groups. 5 groups of that were transplanted with HCC tumor cell, built as HCC mice model. One group of HCC mice were treated with CTX as chemotherapy group when the other three were done with different dose level (7.5 g, 15 g and 30 g/kg/day) of JBDT. After 16 days treatment, tumor tissue were taken out, weighed and compared among groups. Data shown in **Table 1** indicated that either JBDT with high dose level or CTX treatment could reduce the weight of tumor tissue to 34.47% and 43.02%, respectively. This result indicated that high dose level of JBDT and chemotherapy drug reached similar inhibitory effect on curing HCC mice. For further exploration, PCNA expression and cell cycle of tumor tissue were evaluated. We found that PCNA expression shown in HCC mice was strongly positive but was weakly positive after CTX treatment. PCNA expression was further reduced as higher dose level of JBDT applied (**Table 2**). Besides, data shown in **Table 3** indicated that the amount of cells in G1 phase in tumor tissue was increased under higher dose level of JBDT or CTX treatment, which reduced the amount of S phase cells. These results showed that JBDT, especially in higher dose level, had similar effect with CTX on inhibiting tumor cell proliferation.

JBDT has no side effect and protect HCC mice comparing with chemotherapy

CTX is a kind of anti-cancer drug which is widely used in cancer treatment. However, CTX has severe and life-threatening adverse effects, including acute myeloid leukemia, bladder cancer, hemorrhagic cystitis, and permanent infertility, especially at higher dose [9].

We applied the same treatment and grouping as above to see the effect of JBDT on HCC mice. Mice were weighed before HCC model establishment and at the 16th day of HCC transplantation. We found that JBDT significantly increased mice weight in HCC model in a dose-dependent manner when CTX decreased that in HCC model (**Table 4**). In addition, spleen and thymus were also taken out and weighed before HCC model establishment and at 16th day of HCC transplanted. Spleen and thymus weight of groups were compared using spleen and thy-

Table 3. Statistics of cell cycle phases after treatment for each group ($\bar{x}\pm s$)

Group	Quantity (n)	G1 (%)	S (%)	G2 (%)
HCC mice group	10	45.37±2.99	21.47±3.15	33.17±1.75
CTX group	10	76.10±1.33$^{\Delta\Delta}$	5.20±1.13$^{\Delta}$	18.73±0.97$^{\Delta\Delta}$
Low dose JBDT	10	56.43±3.73$^{\Delta}$	13.40±6.93	30.17±4.57
Middle dose JBDT	10	61.07±3.61$^{\Delta\Delta}$	11.00±3.95	27.77±2.74
High dose JBDT	10	70.97±5.75$^{\Delta\Delta}$	7.37±3.98$^{\Delta}$	21.93±1.80$^{\Delta\Delta}$

$^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$, comparison against HCC mice group, two-tailed *t* test.

Table 4. Statistics of body weight with treatment for each group ($\bar{x}\pm s$)

Group	Quantity (n)	Initial weight (g)	Ultimate weight (g)	Difference (g)
Healthy control	10	23.21±1.98	25.90±2.08	2.75±0.46**
HCC mice group	10	23.80±2.15	26.12±3.27	2.40±0.78**
CTX group	10	23.15±2.27	24.05±4.57	1.53±1.23
Low dose JBDT	10	22.90±2.16	28.46±2.01	5.60±1.18**
Middle dose JBDT	10	23.67±1.56	30.03±2.07	6.38±3.81**
High dose JBDT	10	23.97±1.52	31.68±1.82	6.71±2.29**

$^{*}P<0.05$, $^{**}P<0.01$, comparison against CTX group, two-tailed *t* test.

mus index (**Table 5**). Data shown in **Table 5** showed that spleen and thymus index of chemotherapy group declined significantly after treatment when JBDT group helped HCC mice return to the level of healthy status. Besides, blood from vein of post-eyeball was obtained before transplantation with HCC tumor cell and at the 16th day of HCC model establishment. RBC and leukocyte were counted after blood collection. After treatment of CTX for 16 days, the amount of RBC and leukocyte were seriously reduced. In contrast, JBDT elevated the amount of RBC and leukocyte even in low dose level (**Table 6**). These results indicated that JBDT had no side effects on weight and could improve immune system and hematopoietic function in HCC mice.

Discussion

The worldwide incidence of HCC exceeds 600,000 patients per year, and is still rising [10]. Although some drugs such as CTX have already been considered as a kind of tumor killer, people are still harassed by its serious side effects. Baihe, Dihuang and Astragalus have been widely used in Asian medicine for more than 2000 years. Previous reports found that the active components in this three raw material for traditional medicine can improve the function of immune system and repress tumor growth [7, 11, 12]. Here we put them together and prepared decoction of the three as JBDT to evaluate the effect on HCC mice. Our present study demonstrated that JBDT can inhibit tumor tissue growth in a dose-dependent way without side effects.

PCNA is a DNA clamp as homotrimer, which acts as a scaffold to recruit proteins and achieves its processivity by encircling the DNA in eukaryotic cells. PCNA is essential for replication and regarded as an evaluation marker for cell proliferation [13]. The rate of cancer growth depends on proliferative activity. Generally speaking, higher activity of tumor proliferation would induce easier infiltration and lymph node metastasis and worse prognosis [14]. Therefore, tumor proliferation activity is one of pivotal indice for evaluating whether a new drug has valid efficacy. We applied three dose levels of JBDT on HCC mice model and assess PCNA protein expression and found that PCNA protein expression were down-regulated in a JBDT dose-dependent manner. Specifically, highest dose level of JBDT group almost has the same inhibitory rate on PCNA expression as CTX group. That means JBDT could achieve the same therapeutic effect with CTX. We also found that either JBDT or CTX treatment made arrest in G1 phase in tumor tissue and prevented cell proliferation. Arrest of cell cycle could delay tumor growth and alleviated pathological process. This suggsted that JBDT did have inhibitory effect on HCC.

CTX is used to treat cancers and autoimmune disorders. However, CTX has severe and life-threatening adverse effects, including acute myeloid leukemia, bladder cancer, hemorrhagic cystitis, immune dysfunction and permanent infertility, especially at higher doses [15]. Thymus is specialized organ of immune system and the main place for differentiation and mat-

Table 5. The effects of different treatment on spleen and thymus of each group ($\bar{x}\pm s$)

Group	Quantity (n)	Spleen weight (g)	Spleen index (mg/g)[a]	Thymus weight(g)	Thymus index (mg/g)[b]
Healthy control	10	0.398±0.132	14.88±0.70*	0.043±0.008	1.56±0.51*
HCC mice group	10	0.356±0.031	13.03±1.41	0.033±0.008	1.47±0.33*
CTX group	10	0.257±0.029	12.68±1.05	0.021±0.010	0.97±0.32
Low dose JBDT	10	0.357±0.044	14.87±0.95	0.036±0.012	1.50±0.65*
Middle dose JBDT	10	0.410±0.027	15.91±1.28*	0.039±0.016	1.52±0.70*
High dose JBDT	10	0.446±0.039	16.90±1.40*	0.046±0.020	1.67±0.57*

*P<0.05, comparison against CTX group, two-tailed t test; a: spleen index = weight of spleen (mg)/body weight (g); b: thymus index = weight of thymus(mg)/body weight (g).

Table 6. The effects of different treatment on each group ($\bar{x}\pm s$)

Group	Quantity (n)	RBC (k/μl)		Leukocytes (k/μl)	
		Before	After	Before	After
Healthy control	10	5.66±0.71	6.79±0.40	9.27±0.29	10.21±0.81
HCC mice group	10	4.98±0.35	5.51±0.09	10.44±0.60	8.14±0.40##
CTX group	10	4.76±0.69	4.50±0.28#	8.69±0.47	6.43±0.57##
Low dose JBDT	10	3.78±0.26	5.36±0.45#	8.77±0.69	16.34±3.2*,ΔΔ,##
Middle dose JBDT	10	3.78±0.21	5.52±0.61	9.40±0.41	25.42±7.96**,ΔΔ,##
High dose JBDT	10	4.98±0.26	7.60±0.78Δ,**	10.45±1.02	28.26±13.19**,ΔΔ,##

#P<0.05, ##P<0.01, comparison against healthy control group, two-tailed t test; ΔP<0.05, ΔΔP<0.01, comparison against HCC mice group, two-tailed t test; *P<0.05, **P<0.01, comparison against CTX group, two-tailed t test.

uration of T cells which plays a key role in cellular immunity and immune regulation. Atrophy of thymus is always accompanied with sparsely lymphocyte of peripheral immune organs and blood and will directly affect immune function. The spleen is the largest lymphoid organ of body and provides main site for residency, proliferation and immune reaction of various immune cells and synthesis of macrophage enhancing hormone. Therefore, we evaluated immune function of HCC mice using indexes of thymus and spleen regarding their close relation with immune system. Our results indicated that CTX decreased thymus and spleen indexes when JBDT increased that significantly, suggesting that JBDT protected and enhanced immune function of HCC mice. Besides, changes in the amount of leukocytes and hematopoietic function are the widely used indicator of the extent of chemotherapy damage. Results in our experiment showed that the amount of RBC and leukocytes in JBDT group increased in a dose-dependent manner comparing with CTX group. Regarding these results together, it could be deduced that JBDT does not cause damage to body but protect the immune system and hematopoietic function without side effects in the process of treatment on HCC mice.

Conclusion

In conclusion, we established a mouse disease model and proved beneficial effects of JBDT on HCC mice. JBDT can inhibit tumor growth in a dose manner growth without side effects even in higher dose. Our data provides important clues for HCC treatment. Clinical efficacy still needs further research.

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Disclosure of conflict of interest

None.

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