Tumor suppression by Tegafur combined with Barbadian in S-180 tumor-bearing mice

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Abstract. The aim of this study was to discuss the antitumor effect of Tegafur combined with Barbadian on S-180 tumor-bearing mice. A murine tumor model was prepared by subcutaneous injection of S-180 sarcoma cells to the armpit of the right limb of healthy female SPF KM mice. The 24 tumor-bearing mice were randomly divided into 4 groups: Combination therapy group of Tegafur and Barbadian, Barbadian group, Tegafur group and normal saline control group. Corresponding test substances were given to each group by intragastric administration, respectively, 0.2 ml/mouse, once/day, continuous 5 days, interval for 2 days, recorded as 1 period, 3 periods were continuously performed. Antitumor rate, immune cells, blood biochemistry and inflammatory mediators and other indexes were then respectively measured. Result showed that the antitumor rate for the Combination group was 78%; Barbadian group, 72%; and Tegafur group, -89%. White blood cells (WBC) in Barbadian group was significantly higher than that in the control group (P<0.01); lymphocytes (LYMPH), Barbadian group was significantly higher than that in the control group (P<0.01), Tegafur group was significantly lower than the control group (P<0.01); monocytes (MONO), all drug groups were significantly higher than the control group (P<0.01); neutrophils (NEUT), combination group (P<0.01) and Barbadian group (P<0.05) were significantly higher than the control group; blood sugar for the combination (P<0.05) and Barbadian (P<0.01) groups were significantly higher than the control group, while the Tegafur group was significantly lower than the control group (P<0.01). Cholesterol and BUN in the Tegafur group were significantly higher than that in control group (P<0.05). For IL-1, the combination and Barbadian groups were significantly higher than the control group (P<0.05), while for IL-6, all the drug groups were significantly higher than the control group (P<0.05). TNF-α in the Tegafur group was significantly lower than that in the control group (P<0.05). In conclusion, the combination of Tegafur and Barbadian has the significant effect of inhibiting mice S-180 sarcoma. The single use of chemotherapeutic drug Tegafur has no significant inhibitory effect on mice S-180 sarcoma. The single use of Barbadian has good antitumor effect and can resist the significant decrease of lymphocytes caused by the chemotherapeutic drug Tegafur. Thus, Barbadian has a good antitumor effect and can protect the immune system of the body, making it a viable treatment option.

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Introduction

The malignant tumor is usually with latent onset and atypical symptoms, and many cases were found in the advanced stage and could not undergo surgery. Therefore, radiotherapy and chemotherapy become the primary forms of treatment for advanced tumor and recurrent tumor (1). Since radiotherapy and chemotherapy inhibit or kill tumor cells, they have toxic action on the normal cells of the body. Therefore, identification of effective, harmful antitumor drugs has become a research hotspot. Barbadian, the national protected variety of traditional Chinese medicine manufactured by Chinese Medicine Factory Co., Ltd. (Xiamen, China) is mainly made up of natural bezoar, snake gall, antelope's horn, pearl, pseudo-ginseng and natural musk with the functions of eliminating dampness and heat, activating blood, relieving internal heat or fever, excitation and pain. Researchers have found that Barbadian exerts a good anti-dullness effect (2) on the adjuvant therapy of cancer.

In clinic, the combination of Barbadian and chemotherapeutic drugs can relieve patients' pain and improve the clinical symptoms, reduce the toxic and side effect of chemoradiotherapy and prolong patients' lifetime through the function of clearing heat and removing toxicity, but the mechanism is undefined (3,4). In this study, the combination of chemotherapeutic drug Tegafur and Barbadian was used to determine the antitumor effect and initial exploration of antitumor mechanism, which can provide reference and basis for further research and clinical application.

Materials and methods

Materials and reagents. A total of 24 healthy female SPF Kunming mice (weight, 18-25 g) were provided by the

Laboratory Animal Center of Shandong Luye Pharma Co., Ltd. (Yantai, China), the animal certificate number of which was SCXK (Lu) 20090009. S-180 tumor strains of mouse sarcoma cells were provided by the Shanghai Cell Institute (Shanghai, China); Barbadian capsules (batch no. GYZZ Z10940006) were provided by the Chinese Medicine Factory Co., Ltd.; Tegafur capsules (batch no. GYZZ H20080802 were provided by Shandong New Era Pharma; RPMI-1640 was provided by Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA); MTT was provided by Sigma-Aldrich (St. Louis, MO, USA); FBS was provided by Sangon Biotech Co., Ltd. (Shanghai, China); DMSO (AR) was provided by Tianjin Kemiou Chemical Reagent Co., Ltd. (Tianjin, China); mice IL-1, IL-6, TNF-α ELISA kits were provided by Bio-Rad Laboratories, Inc. (Hercules, CA, USA). The study was approved by the Ethics Committee of Yantaishan Hospital (Yantai, China).

Instruments and equipments. The following instruments and equipment were used: Super clean bench CW-CJ-2D [Purification Equipment (Suzhou) Co., Ltd., Suzhou, China]; CO_2 constant temperature incubation (Sanyo Electric Co., Ltd., Osaka, Japan); fully automatic enzyme immunoassay analyzer EVOLIS (Bio-Rad Laboratories, Inc.); fully automatic five classification hematology analyzer LH750 (Beckman Coulter, Inc., Brea, CA, USA); fully automatic biochemical analyzer DXC800 (Beckman Coulter, Inc.); electronic scale YP10002 (Shanghai Youke Equipment & Instrument Co., Ltd., Shanghai, China); electric-heated thermostatic water bath HHS-21-6 (Changzhou Nuoji Instrument Co., Ltd.); biochemical incubator DNP-9002BS-III (Shanghai Cimo Medical Devices Manufacturing Co., Ltd.).

Drug preparation

Preparation of Tegafur. The dose of Tegafur for human is 2 mg/kg, according to the conversion formula of animal dose: DB = DA x RB/RA x (WA/WB)1/3 (DA and DB are the doses of per kg of AB two kinds of animals, RA and RB are the shape coefficients of AB two kinds of animals, WA and WB are the standard weights of animals) (5,6). The Tegafur dose for the mice was 12.28 mg/kg, prepared with sterilized saline and stored at 4˚C in a refrigerator for standby application.

Preparation of Barbadian. The dose of Barbadian for human is 20 mg/kg. For mice, the dose (5,6) of Barbadian was calculated according to the above conversion method, 122.8 mg/kg, prepared with sterilized saline and stored at 4˚C in a refrigerator for standby application.

Preparation of mixed liquor of Tegafur and Barbadian. Sterilized saline was used to prepare the mixed liquor of Tegafur and Barbadian with a final concentration of 24.56 and 245.6 mg/kg.

Preparation of mouse tumor models and group administration

Preparation of mouse tumor models. S-180 myeloma cells of mice in the logarithmic phase were selected and prepared in single-cell suspension with the concentration of $1x10^7$ cell/ml with normal saline under aseptic condition, and injected into the armpit of the right limb of the mice by subcutaneous injection, 0.2 ml/mouse (7-9).

Group administration and record of observation index. The day of subcutaneous inoculation of tumor mass was recorded as 0 day. On the 7th day after the inoculation of tumor mass, the subcutaneous tumor could be touched by hand and the diameter was 0.2-0.4 cm. The group experiment was started, and the mice (n=6 per group) were randomly divided into 4 groups, i.e., combination therapy group, Tegafur group, Barbadian group and normal saline control group. Corresponding test substances were given to mice in each group by intragastric administration, 0.2 ml/mouse, once/day, for an interval for 2 days after continuous administration for 5 days, recorded as 1 period, 3 periods were subsequently performed. The appearance characteristics of mice were recorded every day.

Determination of experimental index

Determination of antitumor rate. Mice were weighed 48 h after the final administration, and then sacrificed. An autopsy was performed, the tumor mass was separated and weighed to calculate the antitumor rate. Inhibition ratio was calculated as: (average tumor weight of control group - average tumor weight of drug group)/average tumor weight of control group x 100% (10-12).

Determination of immune cells. Forty-eight hours after the final administration, the eyeballs were removed to collect 0.2 ml anticoagulant, and the five classification method was used to determine hemocyte. The five classification method refers to the results of five common types of white blood cells in peripheral blood fluid, namely, the percentage and absolute value of eosinophils (EOS), basophils (BASO), neutrophils (NEUT), monocytes (MONO) and lymphocytes (LYMPH), analyzed by the hemocyte analyzer through physicochemical techniques (13).

Determination of blood biochemistry and the level of inflammatory mediators. Forty-eight hours after the final administration, the eyeballs were removed to collect 0.8-1 ml blood, indoor solidification for 30 min, and the supernatant was collected after centrifugation (5,000 x g at 20˚C for 5 min). The ELISA kit was used to determine IL-1, IL-6, TNF-α and other indexes, and a fully automatic biochemical analyzer DXC800 (Beckman Coulter, Inc., Brea, CA, USA) was used to determine all the indexes (14-17) of blood biochemistry.

Statistical analysis. Excel was used in recording the statistical treatment. Data were presented as mean ± standard deviation (mean ± SD). An F test was used to determine homogeneity of variance, t-test was used to determine homoscedasticity or heteroscedasticity according to homogeneity of variance. The difference was statistically significant when $P<0.05$ (18-20).

Results

Effect of the combination of Tegafur and Barbadian on the bodies of tumor-bearing mice. At the end of the experiment, the condition of mice was good according to the visual inspection of combination therapy group (Fig. 1A), Barbadian group (Fig. 1B), normal saline group (Fig. 1D); the fur of mice in Tegafur group (Fig. 1C) were sparse, rough and dispirited (Fig. 1).

Effect of the combination of Tegafur and Barbadian on the tumors of tumor-bearing mice. Tumor weight of the combination therapy and Barbadian groups was significantly

Table I. The effect on the volume of mice tumor by the Tegafur combined with Barbadian.

Group	No. of animals	Tumor volume (mm^3)
Normal saline group	6	230.02±31.25
Combination therapy group	6	112.21±15.65[a]
Barbadian group	6	130.56±13.22[a]
Tegafur group	6	258.51±65.12[a]

[a]P<0.05.

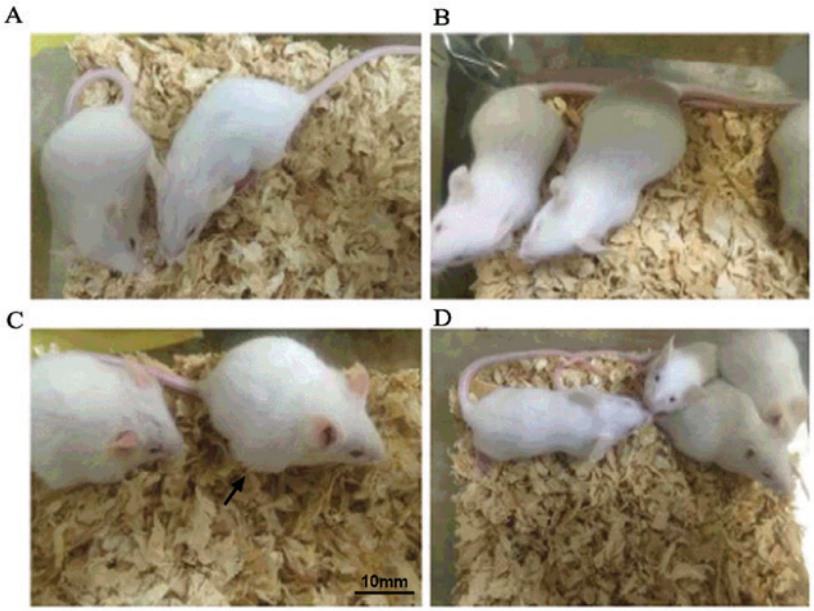

Figure 1. Signs of mice in each group at the end of the third course. (A) Combination therapy group: The coat colour was bright and smooth. (B) Barbadian group: The coat colour was bright and smooth. (C) Tegafur group: The mice hair was sparse, rough and spirits sagged. (D) Normal saline group: The coat colour was bright, smooth and lively. The arrow indicates the tumor of underarms.

lower than that of the normal saline group (P<0.05). Tumor weight of the Tegafur group was higher than the normal saline group, but there was no significant difference (P>0.05). The antitumor rate of the combination therapy and Barbadian groups were 78.00 and 72.64%, respectively. The antitumor rate of the Tegafur group was -89.00%, and the tumor in this group was bigger than that in the normal saline control group (Table I and Fig. 2).

Effect of the combination of Tegafur and Barbadian on immune cells of mice. According to the statistical analysis on immune cells in the report of blood routine test, there was no significant difference in the comparison of the total number of red blood cells in the three drug groups and normal saline group: WBC in the Barbadian group was significantly higher than that in the normal saline group (P<0.01); LYMPH in the Barbadian group was significantly higher than that in the normal saline group (P<0.01), LYMPH in Tegafur group was significantly lower than that in normal saline group (P<0.01); MONO in the three drug groups was significantly higher than that in the normal saline group (P<0.01); NEUT in the combination therapy (P<0.01) and Barbadian (P<0.05) groups was significantly higher than that in the normal saline group.

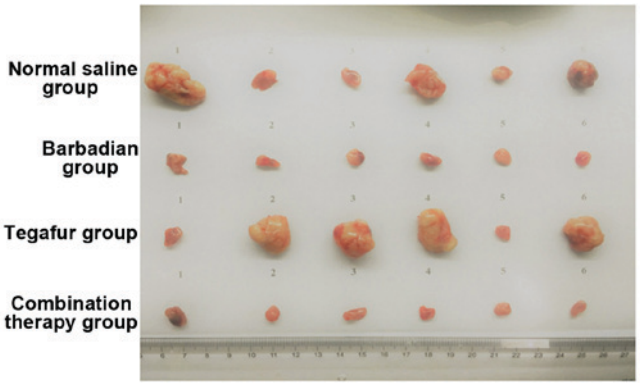

Figure 2. The tumor dissection diagram from 4 different groups.

No significant difference was found in EOS in the three drug groups and normal saline group. BASO in the three drug groups was significantly higher than that in normal saline group (P<0.01); the other indexes were normal (Table II).

The effect of Tegafur combing with Barbadian on inflammatory mediator of mouse. IL-1 in combination therapy group

Table II. The effect of Tegafur combined with Barbadian on the immune cells of mouse (mean ± SD, n=6).

Group	RBC ($x10^{-12}$/l)	WBC ($x10^{-9}$/l)	LYMPH ($x10^{-9}$/l)	MONO ($x10^{-9}$/l)	NEUT ($x10^{-9}$/l)	EOS ($x10^{-9}$/l)	BASO ($x10^{-9}$/l)
Normal saline group	7.65±0.70	3.48±0.77	2.39±0.11	0.62±0.03	0.25±0.03	0.04±0.013	0.085±0.008
Combination therapy group	7.99±0.36	3.54±0.35	2.31±0.39	0.83±0.08[b]	0.35±0.04[b]	0.02±0.013	0.005±0.005[b]
Barbadian group	7.75±0.33	4.76±0.45[b]	3.26±0.29[b]	0.89±0.06[b]	0.31±0.02[a]	0.02±0.017	0.040±0.013[b]
Tegafur group	8.04±0.51	3.37±0.47	1.65±0.36[b]	0.85±0.07[b]	0.23±0.03	0.04±0.013	0.033±0.010[b]

Compared with normal saline group, [a]P<0.05; [b]P<0.01. SD, standard deviation.

Table III. The effect of Tegafur combined with Barbadian on inflammatory mediator of mouse (mean ± SD, n=6).

Group	IL-1 (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)
Normal saline group	3.47±0.80	3.00±0.64	44.00±9.65
Combination therapy group	4.69±0.56[a]	3.98±0.55[b]	34.33±6.31
Barbadian group	4.82±1.60[a]	3.58±0.35[a]	38.17±8.91
Tegafur group	3.90±1.78	3.8±0.57[a]	28.83±9.15[a]

Compared with normal saline group, [a]P<0.05; [b]P<0.01. SD, standard deviation.

Table IV. The effect of Tegafur combined with Barbadian on the blood biochemistry of mouse (mean ± SD, n=6).

Group	FBG (mmol/l)	Total cholesterol (mmol/l)	BUN (mmol/l)
Normal saline group	4.27±0.24	1.83±0.02	7.46±0.54
Combination therapy group	5.43±0.93[a]	1.98±0.73	8.02±0.75
Barbadian group	5.62±0.96[b]	1.88±0.17	7.77±0.31
Tegafur group	2.60±0.35[b]	2.15±0.28[a]	9.68±0.98[b]

Compared with normal saline group, [a]P<0.05; [b]P<0.01. SD, standard deviation.

and Barbadian group were significantly higher than that in normal saline group (P<0.05). IL-6 in combination therapy group (P<0.01), Barbadian group (P<0.05) and Tegafur group (P<0.05) were significantly higher than that in the normal saline group. TNF-α in the Tegafur group was significantly lower than that in normal saline group (P<0.05) (Table III).

Effect of Tegafur combined with Barbadian on blood biochemistry of mouse. According to the statistical analysis on 15 test indexes (including total protein, albumin, globulin, ratio of albumin to globulin, GPT, GOT, GPT/GOT, ALP, g-GGT, TBil, DBil, total cholesterol, triglyceride, BUN, uric acid) of blood biochemistry, there was a significant difference in three test indexes for all the drug groups. The details are as follows: FBG in combination therapy group (P<0.05) and Barbadian group (P<0.01) were significantly higher than that in normal saline group, FBG in Tegafur group was significantly lower than that in normal saline group (P<0.01). Total cholesterol in the Tegafur group was significantly higher than that in normal saline group (P<0.05). BUN in the Tegafur group was significantly higher than that in the normal saline group (P<0.05) (Table IV).

Discussion

We know that chemotherapeutic drugs have a decisive effect on antitumor treatment, but the severe adverse reactions, such as myelosuppression, gastrointestinal reaction, liver and kidney injury have restricted its effect and application to a certain extent (21). Tegafur capsule is a kind of new-style fluorouracil oral anticancer drug, which is made up of FT, CDHP and OXO (22). Its active constituent FT has good oral bioavailability, and can be translated into fluorouracil in the body. CDHP can restrict the decomposition of fluorouracil to make the stability of blood concentration longer in the plasma and tumor tissues to strengthen antineoplastic activity. OXO is distributed in the gastrointestinal tract after oral administration, which can reduce the toxicity and adverse reactions of fluorouracil in the gastrointestinal tract (23). In this study, according to the analysis on immune cells, there was no

significant difference of WBC, RBC, NEUT and EOS in the Tegafur and normal saline control groups. MONO and BASO were increased compared to the normal saline control group, and results were statistically significant. By contrast, LYMPH was significantly reduced compared with the normal saline control group. According to the analysis on cell factors, there was no significant difference of IL-1 in the Tegafur and normal saline groups, while IL-6 was significantly higher than that in normal saline group. TNF-α was significantly lower than that in the normal saline group. According to the analysis on blood biochemistry, blood sugar in Tegafur group was significantly reduced, whereas cholesterol and BUN were significantly increased. The above results show that when Tegafur kills tumor cells, the dead and injured tumor cells cause a series of immune responses through the damage associate molecular pattern (DAMP). When the immune response is started by DAMP and converted from innate immunity into adaptive immune response, rapid division and proliferation focused on T lymphocytes occurred, by this time, the application of Tegafur restricted the DNA composition, when the speed of Tegafur killing lymphocytes is faster than that of oncocytes, the reduced T lymphocytes cannot restrict and kill tumors, so that the result of the tumor growth is increased (antitumor rate is -89%) and higher than normal saline group is obtained.

In this study, Barbadian has good antitumor effect without significant toxic and side effects. In this study, WBC, TLC, MONO, NEUT and BASO in Barbadian group were higher than that in the normal saline group. IL-1 and IL-6 were significantly increased, while TNF-α had no significant difference compared with the normal saline group. Blood sugar was significantly higher than that in the normal saline control group, and there was a significant difference of cholesterol and BUN compared with control group. From the above analysis, it has been shown that the Barbadian group has the significant function of increasing immunity, compared with Tegafur group. The most significant difference was that WBC and LYMPH were significantly increased, while TNF-α had no significant decrease. Additionally, the main drugs of Barbadian including bezoar, pseudo-ginseng, musk and pearl have an immunomodulatory effect; bezoar 100 mg/kg can significantly strengthen the phagocytic function (1) of mouse peritoneal macrophages (MPM), pseudo-ginseng can strengthen NK cell viability and facilitate the activity of macrophage, increasing the lethality (2) of tumors. The study by Hao *et al* (24) has shown that sanchinoside 160 mg/kg can increase 92.0% of hemolytic plaque and significantly increase the phagocytic rate and phagocytic index of MPM. Hao *et al* have shown that pearl has good immunologic enhancement, antitumor and radiation-proof effect (24). Those authors have shown that, Barbadian has the effect of increasing the immunity of the organism. This is in agreement with our result of the antitumor rate of Barbadian group, which reached 72.68%.

The combination of Tegafur and Barbadian has shown the best antitumor rate at 78% in this study. In addition, MONO, NEU, and BASO in combination therapy group were higher than the normal saline control group, and no significant difference is found in other cells compared with the normal saline control group. IL-1 and IL-6 of combination therapy group were significantly higher, while TNF-α had no significant difference compared with the normal saline group. Blood sugar in the combination therapy group was significantly higher than that in the normal saline control group, no difference is found in cholesterol and BUN compared with control group. The above analysis revealed that, compared with Tegafur group, combination therapy group has a milder inflammatory response, the most significant difference being for LYMPH, whereas TNF-α was not lower than the normal saline group and without a significant difference, with the antitumor rate reaching 78%. The possible reason may be the effect of protecting liver and gallbladder of Barbadian has accelerated the metabolism of Tegafur and reduced the toxic effect, improved Tegafur-induced decrease of blood glucose, leading to cholesterol and BUN recover. The key point is LYMPH and TNF-α both returned to normal as indicated by the body hair of mice in the combination group which was glossy and smooth, the mental status of mice was good, and a significant difference was identified compared with the toxic mice in the Tegafur group. Tegafur combined with Barbadian complement each other, they can relieve the toxic and side effects of chemotherapeutic drugs thereby inhibiting the tumor and reaching the desired effect.

In conclusion, this study has shown that the combination of Tegafur and Barbadian has a significant effect of inhibiting mice S-180 sarcoma. The single use of chemotherapeutic drug Tegafur has no significant inhibitory effect on mice S-180 sarcoma, whereas the single use of Barbadian has good antitumor effect and can resist the significant decrease of lymphocytes caused by chemotherapeutic drug Tegafur. Barbadian therefore, not only exerts a good antitumor effect but can also protect the immune system of the organism, which has a good development prospect.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

EZ contributed to the study design, data acquisition and analysis and drafted the manuscript; AF was involved in data acquisition and revision of the manuscript; WC and XW assisted in the performance of the statistical analysis with constructive discussions. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Yantaishan Hospital (Yantai, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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