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Experimental Research

Impacts of Fuzhengxiaoyan decoction on immune functions in nude mice bearing EMT-6 breast cancer

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Summary

Objective: The aim of this study was to observe the impacts of Fuzhengxiaoyan decoction (FZXY) on the immune functions in nude mice bearing EMT-6 breast cancer (EMT-6-carryring mice). *Materials and Methods:* The successfully prepared EMT-6 breast cancer-bearing nude mice models were randomly divided into three groups (n=20): the treatment group: orally administrated FZXY, the control group: orally administrated the Shenmai injection, and the model group: orally administrated an equal volume of normal saline, twice a day for consecutive 14 days. *Results:* The mice in each group were then sacrificed and their spleen and thymus indexes were compared; ELISA was performed to detect the content changes of TGF-β and IL-6 in the spleens. FZXY could significantly increase the spleen and thymus indexes in these EMT-6-carrying mice when compared with the model group ($p < 0.05$); meanwhile, the contents of IL-6 and TGF-β in the spleens of the FZXY group were significantly decreased ($p < 0.05$), similar to those in the positive control group (Shenmai injection). *Conclusion:* FZXY could improve the immune functions of EMT-6- bearing mice, which was the mechanism of this prescription towards inhibiting breast cancer.

Key words: Fuzhengxiaoyan decoction; Spleen index; Thymus index; IL-6; TGF-β.

Introduction

Breast cancer is a major global threat to women's health, its incidence is increasing annually, along with its morbidity and mortality in China, but its survival rate remains low [1, 2]. In recent years, individualized comprehensive treatments of breast cancer have been widely used in clinics [3-5], and traditional Chinese medicine (TCM) begins to play increasingly important roles. The TCM prescription (Fuzhengxiaoyan decoction, FZXY) used in this study is an effective prescription and has been used in clinics for many years, which exhibits significant effects in improving immunity and inhibiting breast cancer. This study explored the mechanism of FZXY, from the aspect of immunology, in intervening breast cancer.

Materials and Methods

Sixty Kunming mice, weighing 18-22 grams, were included in this study. The study also included the following: EMT-6 murine breast cancer cells, mouse IL-6 Elisa detection kit, mouse TGF-β Elisa detection kit, and FZXY. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was initially reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Heilongjiang University.

The cryopreserved EMT-6 murine breast cancer cells were recovered in 37°C water bath, suspended, and centrifuged for subculturing. The EMT-6 cells in the logarithmic growth phase were then sampled and digested into the single cell suspension; after centrifugation, the supernatant was discarded, and the cell density was adjusted to 107 /ml, followed by subcutaneously injecting 0.2 ml into the armpit of each mouse.

After successfully preparing the models, the breast cancer-bearing mice were randomly divided into three groups (n=20). The treatment group was orally administrated FZXY (0.15 ml/10 grams, the specific concentration was calculated according to the "Animal and human per kilogram body weight-dose conversion factor table"), the control group was orally administered the Shenmai injection (0.15 ml/10 grams), and the model group was orally administrated an equal volume of normal saline, twice a day for consecutive 14 days.

Each mouse was weighed before the last medication, and then sacrificed two hours after the last medication; the thymus and spleen were then removed, and weighed on an electronic analytical balance after cleaned residual blood with filter paper, thus obtaining the weights of the thymus (mg) and spleen (mg). The thymus and spleen indexes in nude mice were then calculated using the following formula:

Thymus index (mg/10 g) = [thymus weight (mg)/body weight (g)] × 10;

Spleen index (mg/10 g) = [spleen weight (mg)/body weight (g)] × 10.

The sampled spleen was soaked in 75% ethanol for three- to five-minute sterilization, and then homogenized and passed

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Table 1. — *Impacts of FZXY on spleen index of tumor-bearing nude mice (x ± s, n=20).*

Group	n	Spleen weight (mg)	Spleen index
Treatment group	20	109.89 ± 21.18$^{\Delta}$	5.74 ± 1.11$^{\Delta}$
Control group	20	203.8 ± 29.98*	11.23 ± 1.28*
Model group	20	80.32 ± 26.50	4.08 ± 1.32

$^{\Delta}$ *Comparison between the treatment group and the model group (p <0.05).*
* *Comparison between the control group and the model group (p <0.05)..*

Table 2. — *Impacts of FZXY on thymus index of tumor-bearing nude mice (x ± s, n=20).*

Group	n	Thymus weight (mg)	Thymus index
Treatment group	20	36.59 ± 14.65$^{\Delta}$	1.92 ± 0.80$^{\Delta}$
Control group	20	45.07 ± 18.83*	2.33 ± 0.90*
Model group	20	28.24 ± 4.26	1.46 ± 0.20

$^{\Delta}$ Comparison between the treatment group and the model group (p <0.05).
* Comparison between the control group and the model group (p <0.05).

Table 3. — *Impacts of FZXY on IL-6 and TGF-β contents in the spleen of tumor-bearing nude mice (x ± s, n=20).*

Group	n	IL-6 (pg/mL)	TGF-β (pg/ml)
Treatment group	20	103.81 ± 13.76$^{\Delta}$	90.65 ± 11.01$^{\Delta}$
Control group	20	82.15 ± 14.65*	78.79 ± 24.63*
Model group	20	134.94 ± 20.29	116.37 ± 23.59

$^{\Delta}$ *Comparison between the treatment group and the model group (p < 0.05).*
* *Comparison between the control group and the model group (p < 0.05).*

through a 200-mesh sieve to produce the spleen cell suspension; after centrifugation at 1,000 r/minute for five minutes, the supernatant was discarded, and the erythrocyte lysate was added, followed by gently pipetting for the uniformity; after reacted on ice bath for five minutes, the mixture was centrifuged at 1,000 r/minute for five minutes. The supernatant-discarded residual was then washed with PBS three times; 10% FBS-containing RPMI1640 medium was then added to suspend the cells, which were then placed into T25 flasks, and cultured in one CO_2 incubator for future use. The aforementioned prepared mouse spleen cell suspension then detected the TGF-β and IL-6 contents according to the instructions of ELISA assay kit.

SPSS16.0 software was used for statistical analysis; the statistical analysis included the *t*-test, and expressed as ± s, with $p < 0.05$ or $p < 0.01$ considered as a statistically significant difference.

Results

FZXY and Shenmai injection both significantly increased the spleen weight and spleen index in EMT-6-bearing nude mice compared with the model group, and compared with the model group, the spleen weight and spleen index in the FZXY group were significantly increased ($p < 0.05$), similar to the positive control drug, shenmai injection (Table 1).

FZXY and Shenmai injection both significantly increased the thymus weight and thymus index in EMT-6-bearing nude mice compared with the model group, and compared with the model group, the thymus weight and thymus index in the FZXY group were significantly increased ($p < 0.05$), similar to the positive control drug, shenmai injection (Table 2).

In the spleen of tumor-bearing nude mice, FZXY and Shenmai injection both significantly reduced the IL-6 and TGF-β contents in the spleen of tumor-bearing nude mice compared with the model group, and compared with the model group, the IL-6 and TGF-β contents in the FZXY group were significantly increased ($p < 0.05$), similar to the positive control drug, shenmai injection (Table 3).

Discussion

Breast cancer belongs to the category of rocky masses in breasts in TCM, and it also could be classified into the category of accumulation pattern/syndrome. “Yizhong Bidu” considered that “patients with this accumulation pattern/syndrome lack righteousness, and then are occupied by pathogenic factors”. Modern pathological science believes that malignant cancers are a kind of disease with systemic deficiency with local excess. Therefore, the treatment of cancers by TCM needs to be based on strengthening the body, thus using sufficient assessment to compare the local excess, and this view was also one of the theoretical basis in this study.

FZXY is an effective prescription for the clinical treatment of breast cancer, especially suitable for patients with post-surgery or post-chemotherapy blood and Qi deficiency; it could promote body recovery, inhibit the tumor recurrence, and improve the life quality of patients. This prescription involves 10 grams of Scutellaria Barbata and 20 grams of Radix Codonopsis as Sovereign Drug, basing on Scutellaria Barbata’s effects of clearing heat and expelling toxins. Radix Codonopsis has neutral nature and sweet flavor, and could invigorate spleen-stomach and replenish Qi. Modern research has shown that it has anti-cancer effects, could enhance immunity, increase the activities of superoxide dismutases, and enhance the abilities of eliminating free radicals, thus exhibiting the dual effects of strengthening body and eliminating pathogenic factors when combined with Scutellaria Barbata. Minister Drug, 10 grams of Pinellia Ternata and 5 grams of Rhizoma Curcumae, could disintegrate blood stasis, remove food retention, promote Qi, and relieve pain; thus it can be used to treat aggregation-accumulation and abdominal masses. Pinellia Ternata and Rhizoma Curcumae have been pharmacologically confirmed to contain anti-tumoral ingredients [6-8]. The above four drugs are then further assisted by 10 grams of Radix Paeoniae Alba (tonifying blood, retaining Yin, and relieve pain), 15 grams of Atractylodes Macrocephala (nourishing Qi, invigorating spleen, and enhancing righteousness), to enhance the effects of strengthening body and eliminating pathogenic factors of the main drugs. Fif-

teen grams of Perlcarpium Citri Reticulatae is used to regulate Qi and alleviate pain, as well as to eliminate dampness and phlegm. Furthermore, 15 grams of Lycium Chinense, 15 grams of Evodia austrosinensis, and 15 grams of Fructus Ligustri Lucidi are used to nourish Yin and reinforce the kidneys; the above drugs co-assist the minister drugs to promote their effects of strengthening the body, thus improving the overall anti-cancer effects. Assistant Drug, 10 grams of Poria Cocos (inducing diuresis and removing edema, and could also enhance the effects of reinforcing Qi and strengthening spleen of Atractylodes Macrocephala, thus assisting to strengthen body and improve immune functions), and 15 grams of Angelica Sinensis (activating blood circulation, dissipating blood stasis, nourishing blood, and activating Qi), would then work together to strengthen the effects of the main drugs.

The experimental results showed that FZXY could significantly increase the spleen and thymus weights in the breast cancer-bearing nude mice compared with the model group ($p < 0.05$), significantly increase the spleen and thymus indexes in these tumor-bearing mice ($p < 0.05$), similar to the effects of positive drug control (shenmai injection). The increasing of spleen and thymus weights indicates an enhancement of the immune responses. Thymus and spleen are important lymphoid organs in vivo, and closely related to the body's immune functions. Thymus could produce lymphocytes and transport them to lymph nodes and spleen, and these lymphocytes play important roles towards the cellular immunity. Spleen is the peripheral lymphoid organ in vivo, containing a large number of T cells, B cells, macrophages, etc. After stimulated by antigens, the activated helper T cells enter the lymphatic follicles to trigger the synergistic effects of T and B cells. FZXY could significantly improve the spleen index and thymus index in these tumor-bearing mice, indicating its effects of improving in vivo cellular immunity and humoral immunity, which might be one of the mechanisms of this prescription in inhibiting breast cancer.

IL-6 is a multifunctional cytokine closely related to the proliferation and invasive growth of a variety of tumors. It mainly regulates the cell cycle-related genes, promotes the angiogenesis in tumors, regulates local inflammation environment, and promotes the self-renewal of cancer stem cells, thus involved in the occurrence and development of tumors [9, 10]. Tumors might also produce IL-6 during their proliferation processes. Studies had shown that the serum IL-6 levels in BC patients were significantly increased, suggesting that endogenous IL-6 could be produced and secreted during the proliferation and invasion of breast cancer cells, so it is one of the hallmarks towards tumors' further deterioration [11, 12]. It also suggested that IL-6 could be used as a means to clinically observe and judge the deterioration of those patients. In this study, the IL-6 level in the FZXY group was decreased significantly compared with the model group ($p < 0.05$), indicating that its breast cancer inhibitory effects were obvious, and this was another anti-cancer mechanism of this prescription.

TGF-β is the TGF-β superfamily that could regulate cell growth and differentiation; under certain conditions, this factor could inhibit the proliferation and effects of a variety of immune cells. Tumor cells could secrete TGF-β and other inhibitory cytokines, thus inhibiting the generation of the body's anti-tumoral immunity. In the early development of breast cancer, TGF-β exhibits the activities of a tumor suppressor, and it could inhibit cell proliferation and induce tumor cell death; however, in the advanced stage of breast cancer, tumor cells could tolerate its anti-mitosis role, hence TGF-β would be switched to promoting the malignant process, including invasion and metastasis of tumor cells [13, 14].

TGF-β could not only remodel extracellular matrixes but also increase cell invasion [15]; meanwhile, it could stimulate the mesenchymal transition of epithelial cells (EMT), therefore playing significant promotive roles in tumor progression. The two-way regulation roles of TGF-β lie in its abilities of being able to inhibit the proliferation and differentiation of T and B lymphocytes, inhibit the cytotoxic activities of NK cells and monocytes, inhibit the synthesis of immunoglobulins, and antagonize the roles of such immune regulatory factors as IL -2, TNFA, IFNC, etc. [16, 17], therefore tumors could evade the body/immune surveillance, and promote tumor formation. Tumor cells could autocrine-produce TGFβ, thus promoting the tumor development. TGFβ could also increase the expression of adhesive molecules and extracellular matrixes, thereby enhancing the metastatic potentials of tumor cells [18, 19]. Studies had shown that the expression of TGF-β was closely related to the breast cancer grading, and played a role in the metastasis of breast cancer [20, 21]. The present results showed that FZXY could significantly decrease the TGF-β content in the spleen of tumor-bearing nude mice compared with the model group ($p < 0.05$), further proving its significant breast cancer inhibitory effects, and it is another anti-cancer mechanism of this prescription.

This study showed that the mechanisms of FZXY in treating breast cancer are mainly achieved through enhancing the immune functions, but the tumor inhibitory mechanisms of TCM are complex, and cannot be fully explained as a single mechanism, therefore other future studies assessing other pathways and mechanisms are needed.

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