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Original Article

Immunomodulatory function of the active ingredients in traditional Chinese prescriptions on early stage esophageal cancer with dysplasia

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Abstract: Objective: To explore the effects of the ingredients in a traditional Chinese prescription on the immune regulation of esophageal cancer in patients with early atypical hyperplasia. Methods: A total of 60 C57BL mice were randomly selected as the positive control group; another 240 C57BL mice were randomly divided into a blank control group (n=30), a 4NQO group (n=60) and an experimental group (n=120). The experimental group was divided into the prevention group which received carcinogens and the prescription composition simultaneously, and the treatment group which received carcinogens first and the prescription composition second. The lymphocyte proliferation responses in peripheral blood and spleen cells were determined, and the changes of $CD4^+$, $CD8^+$, B7H4, $CD4^+CD25^+$ before and after administration were measured. Results: The $CD4^+$ cell concentration in the 4NQO group was significantly higher than that in the other four groups ($P<0.05$). No significant difference was found in the $CD4^+B7H4^+$ cell concentration among the five groups ($P>0.05$); the $CD8^+$ concentration in the 4NQO and the prevention groups was significantly higher than that in the other three groups ($P<0.05$). Conclusion: The composition of traditional Chinese prescriptions can improve antitumor immunity and slow down the development of esophageal precancerous lesions by inhibiting the expression of T cells on lymphocytes and the positive expression of B7H4 and $CD4^+$ in esophageal cancer.

Keywords: Traditional Chinese prescription composition, esophageal cancer, peripheral blood, early dysplasia, immune regulation

Introduction

Symptoms are subtle in early stage esophageal cancer, and most patients are diagnosed in advanced stages, and the prognosis by then is seriously affected [1]. The occurrence of malignant tumor lesions is a very complicated process, including tumor formation, proliferation and metastasis, and is often accompanied by gene mutations, angiogenesis, cell apoptosis and the lesion metastasis. Therefore, a large amount of protein substances and cytokines are involved in the occurrence of tumors [2].

Currently, there are many factors affecting the occurrence and development of tumors, but the specific mechanism is still unclear. Lymph node metastasis or infiltration are two biological characteristics of malignant tumors clinically, and are the key factors affecting the clinical prognosis and treatment. In the proliferation and metastasis of tumors, they are positively correlated with human adhesion factors and matrix degrading enzymes [3]. According to traditional Chinese medicine, esophageal cancer is caused by the weakness of spleen and stomach, the dysfunction in acting as a pivot, and disorder of the spleen transformation and transportation; therefore, esophageal cancer originates from the retention of phlegm and blood stasis [4].

In recent years, Chinese scholars have made great progress in the study of tumor differentia-

tion in view of traditional Chinese medicine, and have gained a deep understanding of the chemical constituents and biological functions of Forsythia, trichosanthin, Codonopsis pilosula, and Chinese silkvine root-bark [5, 6]. In this manuscript, forsythiaside, trichosanthin, Chinese silkvine root-bark, Codonopsis pilosula polysaccharides, *etc*. were configured with a ratio of 3:2:2:1 as a prescription to select the optimal treatment in animal experiments, and the effects on precancerous lesions, cell differentiation and immune regulation were explored.

Methods and materials

Animal preparation

A total of 270 C57BL mice, aged 2-6 weeks, with an average age of (4.0 ± 3.5) weeks, including 152 female mice and 118 male mice, weighing 18-20 g, with an average weight of (18.7 ± 3.5) g were purchased. All mice were acclimated for 1 week, and there was no significant difference in age, weight and health status (P>0.05). Each procedure was approved by the Animal Care and Use Committee of Affiliated Hospital of Nantong University.

Experimental drugs

Yellowish-brown plates of 4-Nitroquinoline 1-oxide (4NQO) were purchased from Shanghai Puzhen Technology Co., Ltd., and configured as 100 μg/ml solution. Methyl cellulose, a white to slightly off-white, fibrous or granular, free-flowing powder, 15-4000 mPa.s was purchased from Renqiu Kaibang Chemical Products Co., Ltd., and configured as 1.5% solution. The composition of the prescription included: 1.3 mg trichosanthin, 2 mg forsythiaside, 1.3 g Chinese silkvine root-bark, and 0.6 mg Codonopsis pilosula polysaccharide. The physical and chemical identification methods and thin layer chromatography methods were used to control the concentration of the active ingredients.

Research methods

Prescription composition

Forsythiaside, trichosanthin, Chinese silkvine root-bark, Codonopsis pilosula polysaccharides, *etc*. were configured with a 3:2:2:1 ratio. These active ingredients were sequentially dissolved in 1.5% methyl cellulose to prepare a mother liquor. Then, 2.5 ml of mother liquor was added with 17.5 ml of methyl cellulose (1.5%) for gavage. The concentration of all-trans-retinoic acid (ATRA) was 0.5 mg/ml, and the dissolution medium was methyl cellulose.

Establishment of animal models

All mice were housed at 22-24°C, 40%-60% humidity, and a 12-hour light/dark cycle for 1 week in the animal experiment center. Five mice with the same gender were kept in each cage. The mice were divided into a blank control group (n=30), a 4NQO group (n=60), a treatment group (n=60), a prevention group (n=60) and a ATRA group (positive control group) (n=60). Intragastric administration of preferably 0.1 ml solution was performed on Tuesday, Wednesday and Saturday each week. The blank control group was raised normally and given methyl cellulose. The 4NQO group was raised normally and given the 4NQO solution (100 μg/ml). After 15 weeks, 4NQO was replaced with sterile water. The treatment group was given the 4NQO solution. When atypical hyperplasia occurred, mice were fed with prescription composition and food. The prevention group was fed with the prescription composition and 4NQO solution at the same time. The ositive control group was fed the 4NQO solution, followed by ATRA gavage at the 15th week.

Model processing

The mice in all five groups were sacrificed by cervical dislocation. At the 9th, 12th and 15th week, 5 mice were killed respectively in the blank control group, 4NQO group and prevention group. At the 15th week, tissues from the 4NQO group were stained with hematoxylin and eosin (H&E) to detect early atypical hyperplasia in esophageal tissues. After 15 weeks, the treatment group and the positive control group were given drugs. Since the 16th week, the 4NQO group started to be treated with distilled water. At the 18th week, 5 mice were sacrificed in the blank control group and 15 mice in each of the other four groups. At the 21st week, 7 mice were sacrificed in the blank control group and 15 mice in the other four groups. All remaining mice were sacrificed at the 24th week.

Extraction of spleen cells

The dead mice were immersed in 75% alcohol. The spleens were removed aseptically and placed in the Petri dish. The spleen was gently

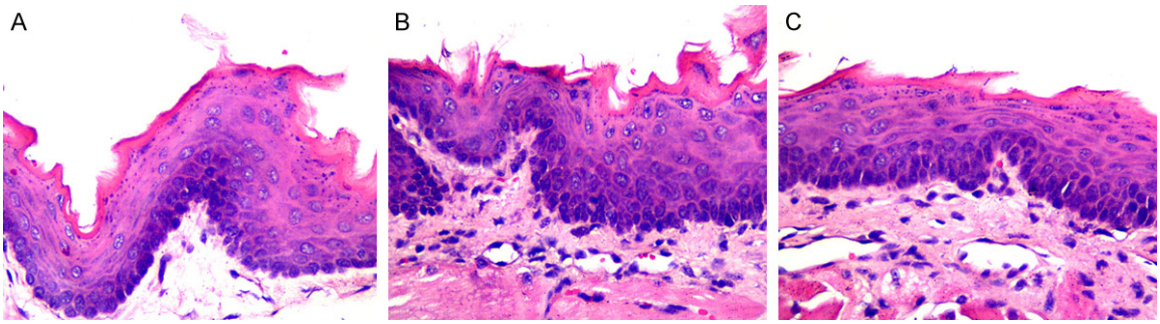

Figure 1. Observation of esophageal histologic section (H&E). A: Mild atypical hyperplasia; B: Moderate atypical hyperplasia; C: Severe atypical hyperplasia.

Table 1. Proportion of $CD4^+$ and $B7H4^+$ cell in each group [%]

Group	$CD4^+$	$B7H4^+$	$CD4^+B7H4^+$	All $B7H4^+$
Blank control group	18.03 ± 8.19	0.52 ± 0.198	0.298 ± 0.199	0.82 ± 0.380
4NQO group	23.45 ± 7.82	0.68 ± 0.85	0.33 ± 0.245	1.01 ± 0.986
Treatment group	17.823 ± 5.57	0.45 ± 0.16	0.157 ± 0.144	0.604 ± 0.256
Prevention Group	21.67 ± 6.196	0.695 ± 0.749	0.27 ± 0.174	0.965 ± 0.765
Positive control group	19.59 ± 6.003	0.627 ± 0.384	0.46 ± 0.485	1.088 ± 0.830
P	<0.05	<0.05	>0.05	<0.05

ground with a 5 ml syringe core to make into a single cell suspension. After filtration through a 200 mesh sieve, the cell suspension was transferred to the centrifuge tube and mixed with 2 ml of lymphocyte separation solution, and then centrifuged (1000 rpm, 5 min) to separate the lymphocytes. The lymphocyte layer was gently collected with pipette into another sterile centrifuge tube and washed twice with PBS. The supernatant was discarded, and 2 ml PBS was added and re-suspended. The cell concentration was adjusted to about 1 * 10^7/ml and labeled with reagents.

H&E staining

The abdomen and thoracic cavity of the sacrificed mice were dissected. The esophagus was exposed, separated, and sliced longitudinally to flatten, which was rolled from the end of esophagus cardia to the end of tongue of esophagus and fixed with a pin. After that, it was placed in the 4% neutral formaldehyde solution and fixed for 24 h. It was dehydrated with 95% ethanol solution and xylene and then embedded with paraffin. After cutting into 3 μm thick sections, the slices were dewaxed with xylene, hydrated with gradient ethanol, stained with H&E, and sealed with neutral balsam. The pathological changes of esophageal tissue were observed under an optical microscope.

Flow cytometry

Esophageal cancer tissues and lesions were detected by intracellular flow cytometry. Through antigens for $CD4^+$, $CD8^+$, B7H4, $CD4^+CD25^+$ in T cells, the numbers of cells such as $CD4^+$, $CD8^+$, B7H4, $CD4^+CD25^+$, *etc*. were calculated. Three tubes were assigned to each mouse. Then, 100 μl of cell suspension and B7H4 antibody were respectively added. CD4 antibody, CD8 antibody, and $CD4^+$, $CD25^+$ antibody were added to tubes 1, 2, 3, incubated at 4°C in the dark for 15 min , washed once with PBS, and centrifuged at 2000 rpm for 5 min. The supernatant was discarded, and 100 μl of PBS was added to each tube, mixed well for testing, and kept in the dark. Groups: ① 100 μl cell suspension + 1 μl $CD4^+$ antibody (0.2 μg) + 400 μl PBS; ② 100 μl cell suspension + 1 μl $CD8^+$ antibody (0.2 μg) + 400 μl PBS; ③ 100 μl cell suspension + 2 μl $CD25^+$ antibody (1 μg) + 400 μl PBS; ④ 100 μl cell suspension + 1 μl B7H4 antibody (0.2 μg) + 400 μl PBS.

Statistical analysis

SPSS 17.0 was used for data analysis. Baseline data were tested by two-sided test. Measurement data were expressed as (x ± sd) and compared by t test, and comparison between the three groups was analyzed by analysis of

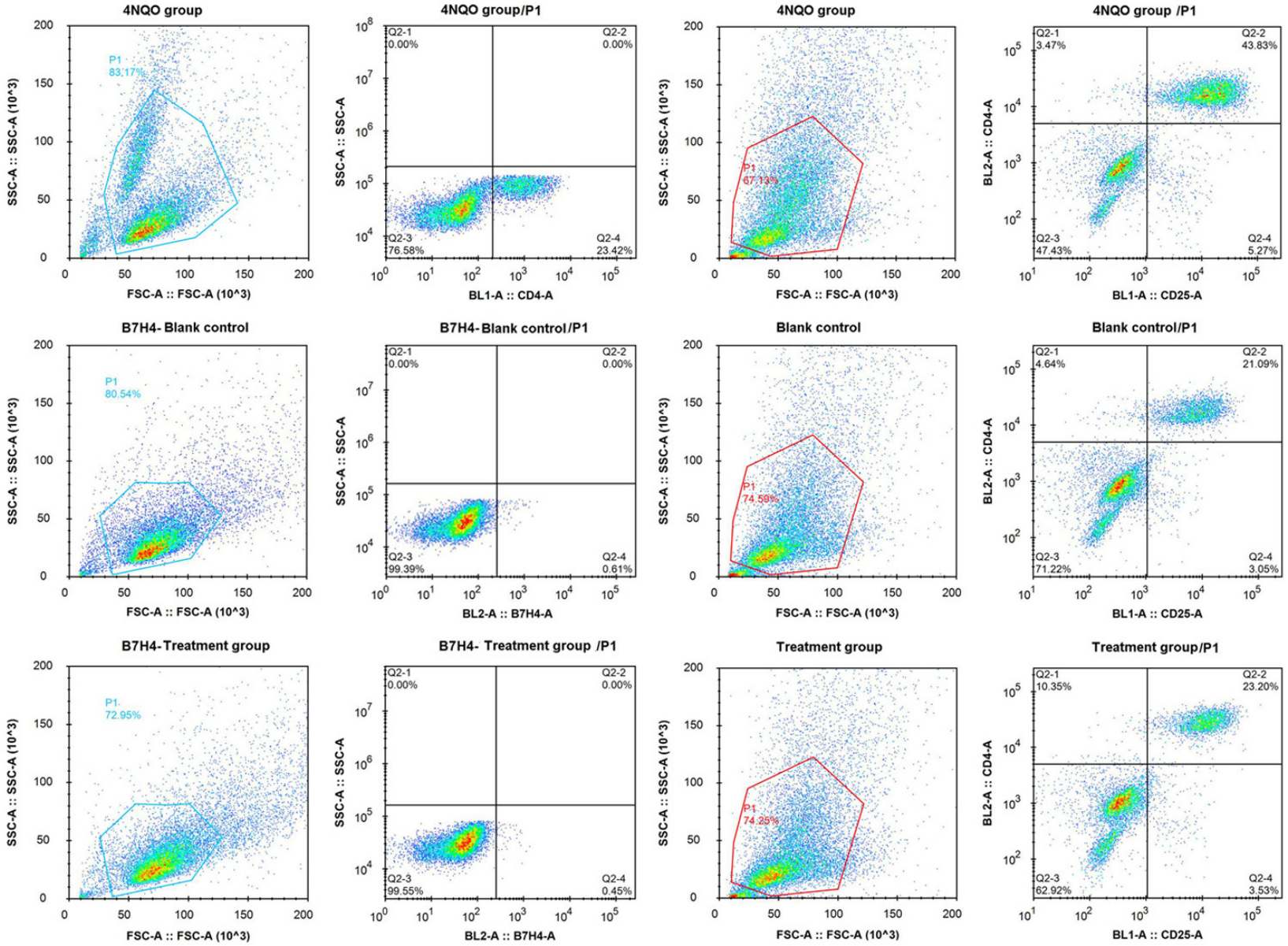

Figure 2. Observation of $CD4^+$ and $B7H4^+$ cells and $CD4^+CD25^+$ detected by flow cytometry.

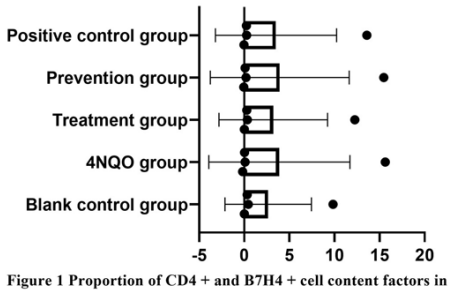

Figure 3. Concentration of $B7H4^+$ and $CD4^+$.

variance, and adjusted using LSD pairwise. Pearson correlation was used for variable association, and chi-squared test was used for count data. $P<0.05$ was considered statistically significant.

Result

H&E staining evaluation

No abnormality was found in the esophagus tissue of the blank control group, which confirmed that methyl cellulose would not lead to biased data. At the 15th week, the 4NQO group showed 1 mild, 1 moderate and 1 severe atypical case of hyperplasia. At the 18th week, the corresponding numbers were 5, 5 and 2, respectively. At the 21st week, those numbers were 1, 3, and 7. There were also 4 cases of esophageal cancer. At the 24th week, there was no mild dysplasia in the 4NQO group, while the numbers of moderate and severe cases were 3 and 11, respectively. It was found that the time of induction was positively correlated with incidence of lesion ($P<0.05$), and it was confirmed that 4NQO can induce mild, moderate and severe dysplasia in esophageal tissue of mice (**Figure 1**).

$CD4^+$ and $B7H4^+$ concentration

The proportion of $CD4^+$ cells in the 4NQO group was significantly higher than that in the other four groups, and there were differences among the five groups ($P<0.05$). The concentration of $CD4^+B7H4^+$ was not statistically significant among the five groups ($P>0.05$). The proportion of all $B7H4^+$ cells in the 4NQN group and the positive control group was significantly higher than that in the other three groups ($P<0.05$, **Table 1**; **Figures 2** and **3**).

$CD4^+$, $CD25^+$, $B7H4^+$ concentration

The number of $CD4^+CD25^+$, $CD4^+CD25^+B7H4^+$ cells in the prevention group was significantly higher than that in the other four groups ($P<0.05$); $CD4^+CD25^+B7H4^+/CD4^+CD25^+$ in the blank control group was significantly higher than that in the other four groups ($P<0.05$, **Table 2** and **Figure 4**).

$CD4^+$, $CD25^+$, $FOXP3^+$ concentration

The number of $CD4^+CD25^+$ cells in the 4NQO group was significantly higher than that in the other four groups ($P<0.05$). The number of $CD4^+CD25^+FOXP3^+$ cells in the prevention group was significantly higher than that in the other four groups ($P<0.05$). The descending order of levels of $CD4^+CD25^+FOXP3^+B7H4^+$ cells in the five groups was the prevention group, treatment group, 4NQO group, blank control group and positive control group, respectively, and the difference was statistically significant ($P<0.05$). The levels of $CD4^+CD25^+FOXP3^+B7H4^+/CD4^+CD25^+FOXP3^+$ in the prevention group were significantly higher than that in the other four groups ($P<0.05$), but the difference was not significant among the 4NQO group, blank control group and treatment group ($P>0.05$, **Table 3**; **Figure 5**).

$CD8^+$ and $B7H4^+$ cell concentration

The proportion of $CD8^+$ cells in the 4NQO group and the prevention group was significantly higher than that in the other three groups ($P<0.05$). The proportion of $B7H4^+$ cells in the blank control group was significantly higher than that in the other four groups ($P<0.05$). The proportion of $CD8^+B7H4^+$ and $B7H4^+$ cells in the blank control group was significantly higher than that in the other four groups ($P<0.05$, **Table 4** and **Figure 6**).

Discussion

Esophageal cancer is commonly diagnosed in the clinic. There are about 259,000 new cases, and 211,000 patients that die of esophageal cancer in China every year, and 90% of esophageal cancers are squamous cell carcinomas. The main treatment option for esophageal cancer is surgery, but the 5-year survival rate of patients with advanced esophageal cancer

Table 2. Concentration of $CD4^+CD25^+B7H4^+$ cells in each group [%]

Group	$CD4^+CD25^+$	$CD4^+CD25^+B7H4^+$	$CD4^+CD25^+B7H4^+$/ $CD4^+CD25^+$ (%)
Blank control group	345 ± 179.098	82.71 ± 53.13	24.83 ± 11.498
4NQO group	529.31 ± 225.24	107.23 ± 95.81	19.81 ± 9.59
Treatment group	383.5 ± 148.39	59.3 ± 32.11	15.54 ± 41.42
Prevention Group	640.14 ± 402.12	137.64 ± 117.14	21.057 ± 10.42
Positive control group	396.22 ± 182.98	58.89 ± 44.83	14.75 ± 5.85

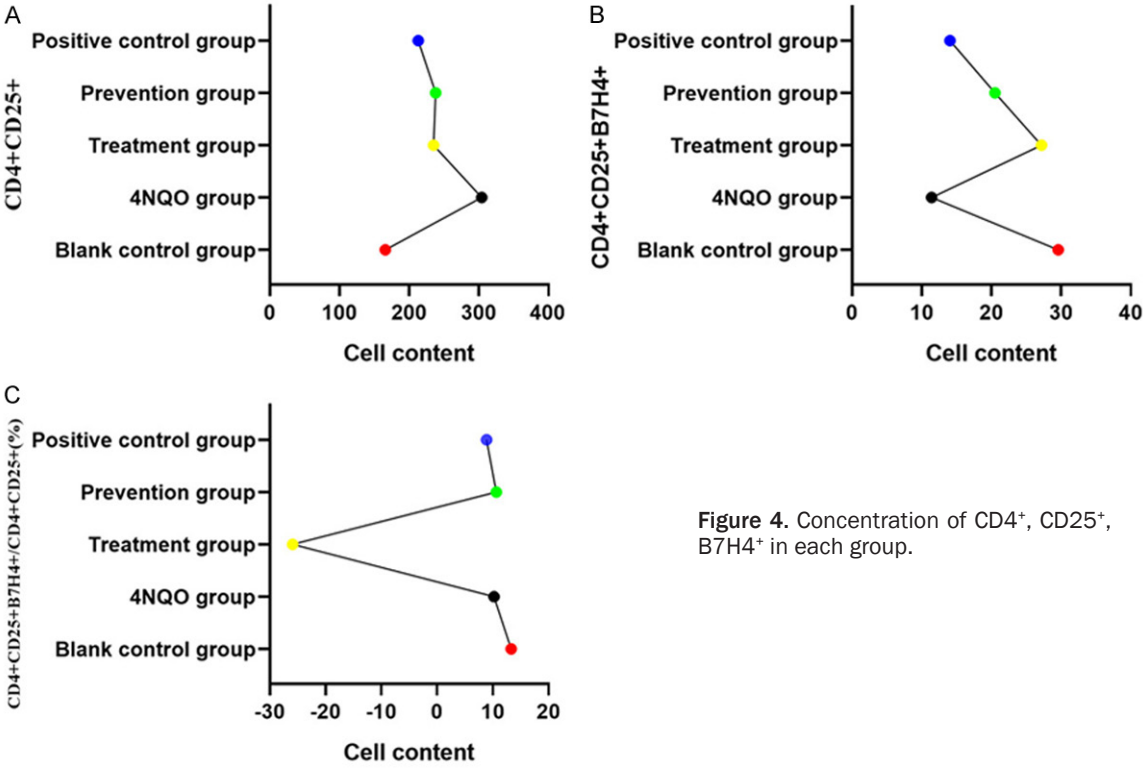

Figure 4. Concentration of $CD4^+$, $CD25^+$, $B7H4^+$ in each group.

Table 3. Number of $CD4^+$, $CD25^+$, and $FOXP3^+$ in each group per 10,000 cells

Group	$CD4^+CD25^+FOXP3^+$	$CD4^+CD25^+FOXP3^+B7H4^+$	$CD4^+CD25^+FOXP3^+B7H4^+$/ $CD4^+CD25^+FOXP3^+$ (%)
Blank control group	32.75 ± 16.96	18.75 ± 15.78	48.64 ± 25.31
4NQO group	43.00 ± 47.35	22.14 ± 26.59	48.84 ± 18.26
Treatment group	46.86 ± 31.92	28.14 ± 27.62	50.62 ± 22.85
Prevention Group	51.11 ± 55.97	33.44 ± 40.67	53.91 ± 30.72
Positive control group	25.00 ± 14.85	14.33 ± 10.19	53.58 ± 14.83
P	<0.05	<0.05	<0.05

that underwent surgery is 20.64%-34.00%. It has been found that the occurrence of esophageal cancer is related to genetic abnormalities. When cancer cells are activated and overexpressed, they promote cell proliferation and differentiation, leading to the loss or inactivation of tumor suppressor genes. Some scholars believe that esophageal cancer is caused by cell apoptosis resulting from genetic abnormalities which promote cell proliferation and malignant transformation, and lead to the proliferation of poorly differentiated cells to form cancer cells

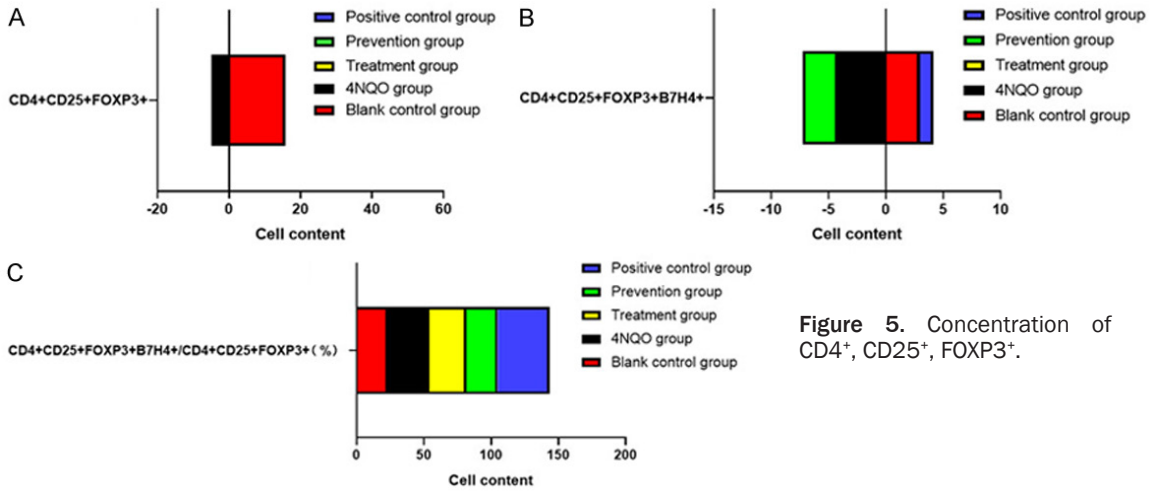

Figure 5. Concentration of $CD4^+$, $CD25^+$, $FOXP3^+$.

Table 4. Concentration of $CD8^+$, $B7H4^+$, and $CD8^+B7H4^+$ cells in each group [n/%]

Group	$CD8^+$	$B7H4^+$	$CD8^+B7H4^+$	All $B7H4^+$
Blank control group	15.45 ± 7.98	0.55 ± 0.37	0.12 ± 0.13	1.30 ± 0.97
4NQO group	16.86 ± 3.03	1.18 ± 0.86	0.053 ± 0.079	0.59 ± 0.36
Treatment group	14.95 ± 3.32	0.48 ± 0.17	0.04 ± 0.031	0.54 ± 0.23
Prevention Group	16.61 ± 8.62	0.645 ± 0.303	0.045 ± 0.304	0.68 ± 0.31
Positive control group	11.38 ± 3.44	0.53 ± 0.19	0.039 ± 0.030	0.57 ± 0.21
P	<0.05	<0.05	<0.05	<0.05

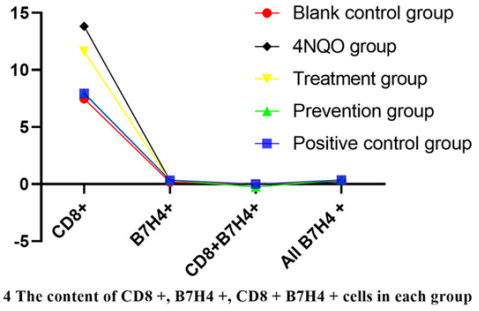

Figure 4 The content of CD8 +, B7H4 +, CD8 + B7H4 + cells in each group

Figure 6. Concentration of $CD8^+$, $B7H4^+$, $CD8^+B7H4^+$ cells in each group.

[7, 8]. Esophageal cancer mainly occurs in the middle-aged and elderly, and the incidence rate increases rapidly around the age of 35-39 years. Deaths between the age of 50 and 75 years old account for more than half of all deaths from esophageal cancer [9, 10]. Surgery is the preferred method for the treatment of early esophageal cancer. For patients who cannot be cured or receive radiotherapy in the advanced stage, esophagogastric bypass surgery, gastrostomy, and esophageal cavity catheterization are usually used to solve dietary problems. Most of the esophagus is resected according to the conditions, and the margins of resection are recoomended to be at least 5 cm away from the tumor [11, 12]. In this study, it was found that 4NQO treatment induced precancerous lesions in the esophageal tissues of mice, and early atypical hyperplasia occurred at the 15th week. With the increase of time, the lesions of the mice in the 4NQO group increased gradually, confirming the successful establishment of the esophageal cancer models. $CD4^+CD25^+$ are regulatory T cells. When tumors develop to advanced stages, $CD4^+CD25^+$-T cells can play an inhibitory role in tumor tissues [13, 14]. The risk factor for esophageal cancer was a high level of nitrosamine compounds in the gastric content, resulting in a strong carcinogenicity. The presence of mold in the body can induce gene mutations and synergistically enhance the carcinogenicity of nitrosamines. Pathological analysis showed that the occurrence of esophageal cancer was a continuous evolution, that is, mild dysplasia → moderate dysplasia → severe dysplasia → carcinoma *in situ* → invasive carcinoma. Atypical hyperplasia of esophageal squamous

epithelium indicated a precancerous lesion stage. If appropriate intervention is given in time, cells in the precancerous lesion stage can be induced to differentiate into normal cells and return to a healthy state [15]. Wolf et al. [16] found that $CD4^+CD25^+$-T cells in peripheral blood of patients with epithelial malignant tumors increased significantly, while T cell proliferation was inhibited.

$FOXP3^+$ is a specific marker in $CD4^+CD25^+$-T cells, and $FOXP3^+$ is expressed in many tumors [17, 18]. This study showed that the expression of $CD4^+CD25^+FOXP3^+$ in the 4NQO group was significantly higher than that in the other four groups ($P<0.05$), suggesting that the traditional Chinese prescription can reduce the incidence of dysplasia by inhibiting the expression of regulatory T cells. Some studies have found that the positive expression rate of regulatory T cells in the development of esophageal cancer in mice is significantly increased, indicating that the positive expression rate of regulatory T cells is significantly correlated with the patient's condition, which is similar to results of other studies [19, 20]. B7H4 is expressed in breast cancer, lung cancer and ovarian cancer, and plays a negative regulatory role in the anti-tumor immune response [21, 22]. Studies have found that dendritic cells (DC), T cells and B cells exhibited B7H4 expression. They exert anti-tumor immune responses by inducing DC cells to produce TGF-β, transformation of T cells into regulatory T cells and induction of $CD8^+$ CTL apoptosis [23-25].

The positive expression of $CD4^+B7H4^+$ in the 4NQO group was significantly higher than that of positive control group, and increased with the extension of induction, suggesting that $CD4^+$ cells may bind to $CD8^+$ cells or B cells through the surface B7H4 molecule, inhibiting $CD8^+$ cell function and promoting immunosuppression. This study found that compared with the 4NQO group, the positive expression rate of B7H4 in $CD4^+$ cells was reduced in the treatment and the prevention groups, indicating that this prescription reversed the immunosuppression in the development of esophageal precancerous lesions and slowed the development the esophageal precancerous lesions.

To sum up, the expression rate of $CD4^+CD25^+FOXP3^+$ cells in lymph was significantly increased during the progression of esophageal precancerous lesions, and the traditional Chinese medicine prescription improved the body's anti-tumor immunity by inhibiting the positive expression of B7H4 in $CD4^+$ cells.

Disclosure of conflict of interest

None.

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