Study of immune responses in mice to oral administration of Flor·Essence

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Received April 15, 2019; Accepted November 18, 2019

DOI: 10.3892/mco.2020.2023

Abstract. Flor·Essence (FE), a natural food grade herbal formula product manufactured by Flora Manufacturing & Distributing Ltd., has been used by patients with cancer in North America to stimulate immune cells in order to attenuate or reverse immune damage. To elucidate the mechanisms underlying the effects of FE on the immune system, spleen lymphocyte proliferation was analyzed by an MTT assay, and the phagocytic capacity of macrophages was measured via the neutral red phagocytosis method. The cytotoxicity of natural killer (NK) cells towards K562 cells was assessed via a CytoTox 96 assay. The production of the cytokines interleukin (IL)-12 and interferon (IFN)-γ in the peripheral blood was determined via ELISA and PCR analysis. The expression levels of caveolin-1 and NF-κB were measured via western blotting. In addition, cyclophosphamide was used to establish a mouse model of immunosuppression. It was found that the proliferation of splenocytes, the phagocytic capacity of macrophages and the cytotoxicity of NK cells against K562 cells were increased after oral administration of FE to mice. FE augmented the production of IL-12 and IFN-γ in the peripheral blood of mice. FE significantly increased the expression of proliferating cell nuclear antigen and caveolin-1, and decreased NF-κB expression. Finally, FE enhanced the viability of immune cells from cyclophosphamide-treated immunosuppressed mice. The results indicated that FE could activate immune responses and enhance natural immunity, suggesting that oral administration of FE can activate the body's immune response and resist damage caused by cyclophosphamide chemotherapy.

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Key words: Flor·Essence, natural killer cell, splenocytes, immunity cytokines, immunity ability

Introduction

Malignant tumors are amongst the leading causes of human mortality (1). The current methods of treating tumors are predominantly radiotherapy and chemotherapy, which improve the therapeutic outcomes of patients with malignant tumors; however, the side effects are a major obstacle in some patients (2).

Numerous patients who do not respond to traditional therapies frequently seek adjuvant and alternative medical treatments in the hope of reducing tumor size and enhancing immunity (3). In Western high-income countries, the use of complementary and alternative medicine (CAM) in patients with cancer is as high as 91% (4,5). Immunostimulants can enhance the body's immunity, and one of the research directions of CAM is to find safe and effective immune stimulants that can be used in clinical practice to prevent diseases (6,7).

Flor·Essence (FE) Tonics is a complex mixture of commercially available herbal extracts, and is sold as a dietary supplement and used by patients with cancer due to reports that it may aid in treating or preventing disease (8). Large quantities of supplements are shipped to the US and Canada annually, primarily for use in cancer treatment (9-13).

FE contains eight different types of herb, which predominantly contain phenolics. The FE formula is proprietary; however, the composition in descending order of magnitude is as follows: i) Burdock root; ii) sheep sorrel herb; iii) slippery elm bark; iv) watercress herb; v) kelp; vi) blessed thistle herb; vii) red clover herb; and viii) Turkish rhubarb root (14). It has been reported that kelp may reduce the incidence of cancer by affecting oxidative activity, specifically in liver cancer and lung cancer (15,16). Arctigenin inhibits mitogen-activated protein kinases, including ERK1/2, p38 kinase and JNK, resulting in an increase in the activity of NAPDH oxidase 2, tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (17). Dietary isoflavones inhibit the progression of prostate cancer by inducing apoptosis in tumor cells (18). It has been reported FE has various positive functions, including antioxidant, antiestrogen, immunostimulatory and antitumor effects (8,19,20). Cheung *et al* (20) reported that FE inhibited nitric oxide (NO) production in a concentration-dependent

manner in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Reverse transcription-quantitative PCR assays revealed that FE induced the expression of IL-1β, inducible nitric oxide synthase and cyclooxygenase-2 in RAW 264.7 cells.

In the present study, an immunosuppressive mouse model was established using cyclophosphamide (CP) to explore the effects of FE on the side effects of chemotherapy.

Materials and methods

Sample preparation. FE was obtained from Shenzhen Fulan Trading Co., Ltd. (Flora Manufacturing & Distributing, Ltd.). FE was boiled with distilled water overnight in a small bowl. Samples were filtered with a 200-μm nylon mesh. Then, it was cooled and refrigerated at 4˚C. Concentration of 10, 20 and 40 mg/kg FE were prepared for oral administration in mice.

Animals and supplementation with FE. Specific pathogen-free Kunming mice (male, 6-8 weeks old, 18-22 g) were supplied by the Experimental Animal Center of Dalian Medical University. The mice were housed under standard laboratory conditions (25˚C, 40-60% relative humidity, under a 12:12 h light/dark cycle) and were fed with standard rodent food and water *ad libitum*. Mice were monitored and weighed daily. A total of 80 mice were randomly divided (20/group) into control group (saline), low-dose FE group (10 mg/kg), medium-dose group (20 mg/kg) and high-dose group (40 mg/kg). FE was administered via gavage twice daily for 5 weeks.

Measurement of immune organs and weight. Mice were sacrificed by CO_2 asphyxiation after a final weight measurement (3 mice/group). The spleen and thymus were removed aseptically, dried and weighed. The organ index was calculated as follows: Thymus index = thymic weight (mg)/animal weight (g) x100%; spleen index = spleen weight (mg)/animal weight (g) x100%.

Spleen lymphocyte isolation. Spleens were aseptically removed and homogenized through 200-μm nylon mesh (three mice per group). To prepare a single cell suspension, red blood cell lysate (Beijing Solarbio Science & Biotechnology Co., Ltd.) was added, allowed to stand for 8 min, and centrifuged at 800 x g for 30 min at room temperature. The lymphocyte layer was aspirated and resuspended in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) for density gradient centrifugation at 250 x g for 10 min at room temperature. The cells were cultured in RPMI-1640 supplemented with 10% FBS (cat. no. 10099141; Gibco, Thermo Fisher Scientific, Inc.). All cells were maintained in a humidified atmosphere at 37˚C in 5% CO_2.

MTT assay for cell viability. Each group of mouse spleen cells were seeded at $4x10^4$/well in 96-well plates and cultured in RPMI-1640 supplemented with 10% FBS at 37˚C with 5% CO_2. LPS (100 μg/ml; cat. no. L2630; Sigma-Aldrich; Merck KGaA) was added to the culture medium for 24 h, following which MTT reagent (5 mg/ml; cat. no. V13154; Gibco; Thermo Fisher Scientific, Inc.) was added to the cell medium and cells were incubated at 37˚C for an additional 4 h. The reaction was terminated with 150 μl/well DMSO and the cells were lysed for 15 min, with the plates agitated every 5 min. Absorbance values were determined using a microplate reader at 492 nm.

Peritoneal cell isolation. Mice were fasted for >8 h before surgery, and mice were sacrificed by CO_2 asphyxiation (3 mice/group). The mice were soaked for 3 min, the skin was cut open and the peritoneum was fully exposed. Pre-cooled 1640 medium (5 ml) was intraperitoneally injected, and the abdomen of the mouse was incubated for 5 min. The peritoneal lavage fluid was withdrawn by a syringe and collected in a 15-ml centrifuge tube; this was repeated twice. The supernatant was centrifuged at 4˚C and 450 x g for 8 min, and red blood cell lysate was added until a white cell pellet was observed. The cells were cultured in RPMI-1640 supplemented with 10% FBS, and then seeded into 96-well culture plates. Cells were maintained in a humidified atmosphere at 37˚C in 5% CO_2.

Phagocytosis neutral red method. Peritoneal macrophages were obtained, 4% trypan blue was mixed with cells at a concentration of 9:1 at room temperature (final concentration, 0.04%), and cells counted after ~3 min and incubated at 37˚C for 2 h to allow cells to adhere. The cells were washed twice with D-Hanks solution (Beijing Solarbio Science & Biotechnology Co., Ltd.) to remove unattached cells. Neutral red (100 μl) was added to each well, and after 4 h at 37˚C, wells were washed 3 times with D-Hanks solution. After adding 100 μl of cell lysate, after 30 min, the optical density (OD) value of each well was measured at 490 nm by a microplate reader.

Semi-quantitative PCR. TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) reagent was used to extract total RNA from spleen lymphocytes. Then, a ProtoScript II RT-PCR kit (Takara Biotechnology Co., Ltd.) (21) was used to reverse transcribe RNA. qPCR analysis of interferon (IFN)-α, IFN-β and GAPDH was conducted using the following gene-specific primers: IFN-α, forward, 5'-ATGAGCACTGAAAGCATGATC-3' and reverse, 5'-TCACAGGGCAATGATCCCAAAGTAGACCTG CCC-3'; IFN-β, forward, 5'-CTTGAATTCACTGCTCTC CTG-3' and reverse, 5'-CGGCTCGAGTCAGTTAGGGAG-3'; GAPDH, forward, 5'-CTAGCACCCCTGGCCAAG-3' and reverse, 5'-GATGTTCTGGAGAGCCCCG-3'. PCR was conducted as follows: An initial denaturation step at 94˚C for 5 min, followed by 30 cycles of annealing for 30 sec, 53˚C for 30 sec and 72˚C for 20 sec, and a final extension step at 72˚C for 10 min. PCR products were resolved via 1% agarose electrophoresis and visualized using ethidium bromide. Quantitative analysis was performed using ImageQuant TL 7.0 software (GE Healthcare Life Sciences).

Lactate dehydrogenase (LDH) assay of the viability of natural killer (NK) cells. Mice were sacrificed by CO_2 asphyxiation and soaked in 75% ethanol, and the spleens of the mice were removed (3 mice/group). Mouse 1X lymphocyte separation solution (4-5 ml; cat. no. P8620; Beijing Solarbio Science & Technology Co., Ltd.) was added to the culture dish for grinding. Immediately, the lymphocyte supernatant containing the spleen cells was transferred to a 15-ml centrifuge tube, 200-500 μl of RPMI-1640 medium was added

(maintaining a clear liquid boundary), the lymphocyte layer was aspirated and a further 10 ml of RPMI-1640 medium was added. The solution was centrifuged at 800 x g for 30 min at room temperature. The cells were collected via further centrifugation at 250 x g for 10 min at room temperature. The supernatant was decanted and the cells were cultured in RPMI-1640 supplemented with 10% FBS. Cells were maintained in a humidified atmosphere at 37˚C in 5% CO_2.

The cytotoxicity of NK cells towards K562 cells (cat. no. BNCC339825; BeNa Culture Collection) was evaluated via co-culture (50:1) under standard conditions for 24 h at 37˚C in 5% CO_2, followed by using a CytoTox 96® LDH assay kit according to the manufacturer's protocol (cat. no. A020-2-2; Nanjing Jiancheng Bio-Engineering Institute Co., Ltd.).

The percentage of cytotoxicity was calculated using the formula below:

$$\% \text{ Cytotoxicity} = (OD_{sample}-OD_{contrast})/(OD_{standard}-OD_{blank})x100$$

Measurement of cytokines. The expression of IL-12, IFN-γ, IL-2 and TNF-α was measured using double antibody sandwich ELISA kits (IL-12, cat. no. BMS6004; IFN-γ, cat. no. BMS228; IL-2, cat. no. BMS601; TNF-α, cat. no. BMS607-3; all from Invitrogen; Thermo Fisher Scientific, Inc.). During euthanasia, blood (0.5 ml) was collected from each mouse (3 mice/group) via retro-orbital sampling, allowed to stand at room temperature for 2 h and at 4˚C for 3-4 h, centrifuged at 800 x g for 5 min at room temperature and stored at -20˚C. The supernatant from each group was collected, and their levels were quantitated by ELISA according to the manufacturer's protocols.

Western blot analysis. Spleens were aseptically removed and homogenized through 200-μm nylon mesh. Equal quantities (100 μg/lane) of spleen protein were analyzed via western blot analysis as previously described (22). Membranes were incubated with primary antibodies at 4˚C overnight, and secondary antibodies [horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG (1:8,000; cat. nos. TA140003 and TA130003, respectively; OriGene Technologies, Inc.)] at room temperature for 1 h. The following primary antibodies were used: Anti-caveolin-1 (Cav-1; mouse monoclonal; 1:1,000; cat. no. 03-6000; Invitrogen; Thermo Fisher Scientific, Inc.); anti-NF-κB (rabbit monoclonal; 1:1,000; cat. no. 8242T; Cell Signaling Technology, Inc.); anti-proliferating cell nuclear antigen (PCNA; mouse monoclonal; 1:500; cat. no. BM0104; Wuhan Boster Biological Technology, Ltd.); and anti-β-actin (mouse monoclonal; 1:1,000; cat. no. BM0627; Wuhan Boster Biological Technology, Ltd.). Bands were visualized using the BeyoECL Plus reagent (cat. no. P0018S; Beyotime Institute of Biotechnology) according to the manufacturer's protocols. Immunoblot data were quantified using ImageJ software (version 1.46; National Institutes of Health).

CP preparation of mouse model of immunosuppression. Specific pathogen-free Kunming mice (male, 6-8 weeks old, 18-22 g) were provided by the Experimental Animal Center of Dalian Medical University. A total of 90 mice were randomly divided (45/group) into a control group and CP group. The 45 mice in each group were randomized into 3 groups (15/group): Control group (saline), low-dose FE group (10 mg/kg) and high-dose FE group (40 mg/kg). Mice were intraperitoneally injected with physiological saline or CP (75 mg/mg; Beijing Solarbio Science & Biotechnology Co., Ltd.) once every other day with CP for 8 days. At 8 days later, immunological indicators of the control and CP groups were measured to verify whether the immunocompromised mouse model was successfully constructed. Then, all mice were intragastrically administered with saline, or 10 or 40 mg/kg FE twice a day for 5 weeks, and the tissues were extracted on the last day.

Determination of the number of white blood cells. During euthanasia, blood (0.5 ml) was collected from each mouse (3 mice/group) via retro-orbital sampling, allowed to stand at room temperature for 2 h and at 4˚C for 3-4 h, centrifuged at 800 x g for 5 min at room temperature and stored at -20˚C. Leukocyte diluent (2.0 ml glacial acetic acid, 1 ml 1% gentian violet, 100 ml distilled water) was prepared and filtered, and 0.19 ml of this solution was added to 20 μl peripheral blood using a hemoglobin pipette. The pipette was used to mix the blood and diluent thoroughly; the micropipette quickly absorbs the mixed suspensions, which was added to a counting cell. After standing for 2-3 min, the cell was microscopically examined; the total number of white blood cells in the four corner squares was counted using an optical inverted microscope (magnification, x40).

Statistical analyses. All data were expressed as the mean ± SE. Unpaired Student's t-tests were used to analyze significant differences between the control and CP groups. Comparisons of multiple groups were analyzed using one- or two-way ANOVA followed by post hoc Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of FE on immune organs, splenocytes and PCNA. The spleen and thymic indexes of the mice supplemented with FE were significantly higher than the control group following 5 weeks of supplementation (Fig. 1A and B). In almost all rapidly proliferating cells, PCNA is important for DNA replication and cell cycle regulation, and is a key factor for measuring cell proliferation ability (23,24). The effects of FE on proliferation in the immune organs were investigated by analyzing PCNA expression in splenocytes. The levels of PCNA in the mice administered with 10 or 40 mg/kg FE were significantly higher than in the control group (Fig. 1C). These results suggested that FE can enhance the viability of immune cells.

Effects of FE on immune cell activity and cytokine production. The macrophages obtained from mice which had been orally administered with FE had significantly increased phagocytic activity against neutral red (Fig. 2A). Mice supplemented with FE exhibited increased NK cytotoxicity compared with the control (Fig. 2B). In addition, FE also induced the production of IFN-α and IFN-β, which were evaluated via PCR. The data demonstrated that spleen lymphocytes from FE-supplemented mice expressed higher levels of IFN-α and IFN-β than those from the controls (Fig. 2C). It was also shown that FE supplementation significantly increased the secretion of IL-12 and IFN-γ into the blood (Fig. 2D).

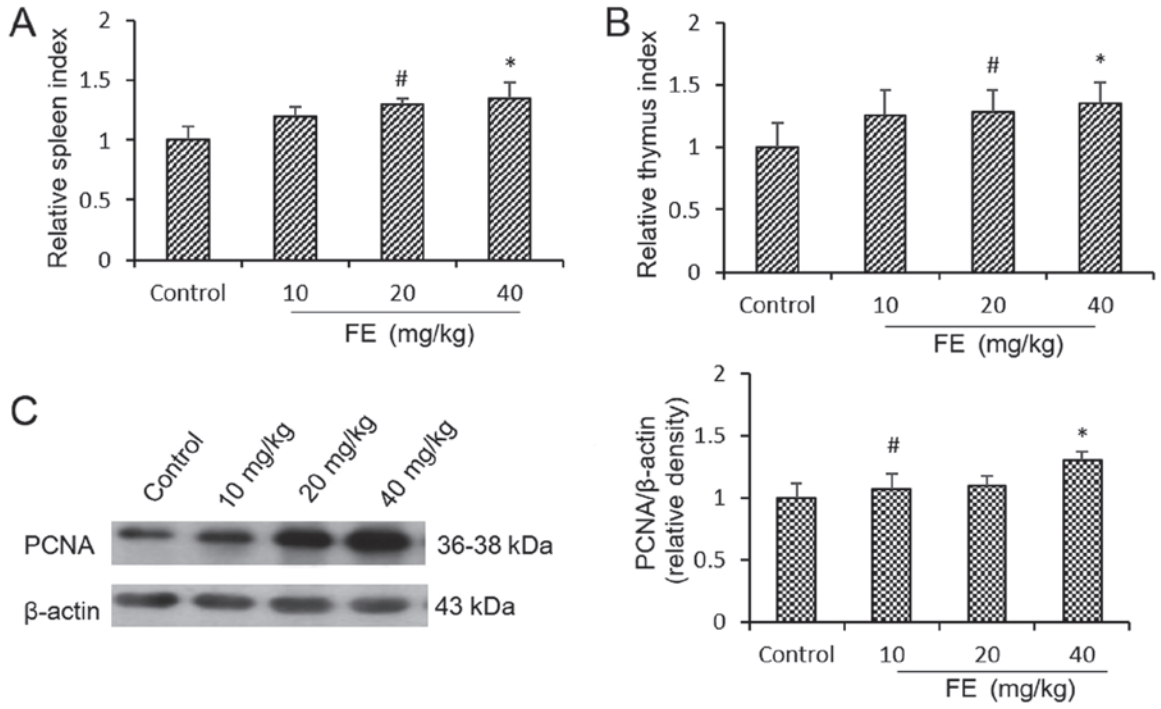

Figure 1. Effects of FE on the immune organs of mice. (A) Spleen index of mice receiving FE by gavage. (B) Thymus index of mice receiving FE by gavage. (C) PCNA levels were significantly higher in the mice that received FE compared with the control (n=3). *P<0.05 and #P<0.05 vs. Control. FE, Flor·Essence; PCNA, proliferating cell nuclear antigen.

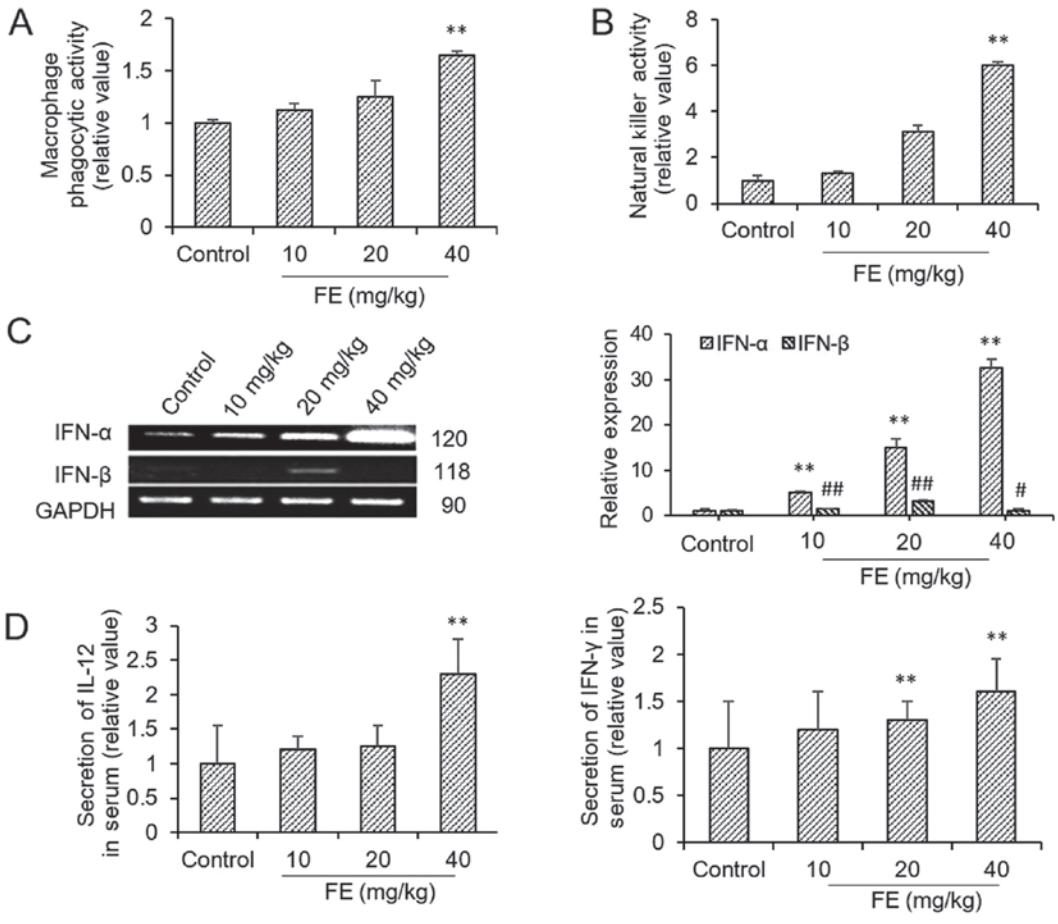

Figure 2. Effects of FE on immune cell activity and cytokine production. (A) Effects of FE on peritoneal macrophage phagocytosis in normal mice. (B) Effects of FE on natural killer cell activity in normal mice. (C) Effects of FE on the expression of IFN-α and IFN-β as determined via PCR. (D) Effects of FE on the secretion of IL-12 and IFN-γ in serum, as measured via ELISA. N=3. **P<0.01 vs. Control (IFN-α); #P<0.05 and ##P<0.01 vs. Control (IFN-β). FE, Flor·Essence; IL, interleukin; IFN, interferon.

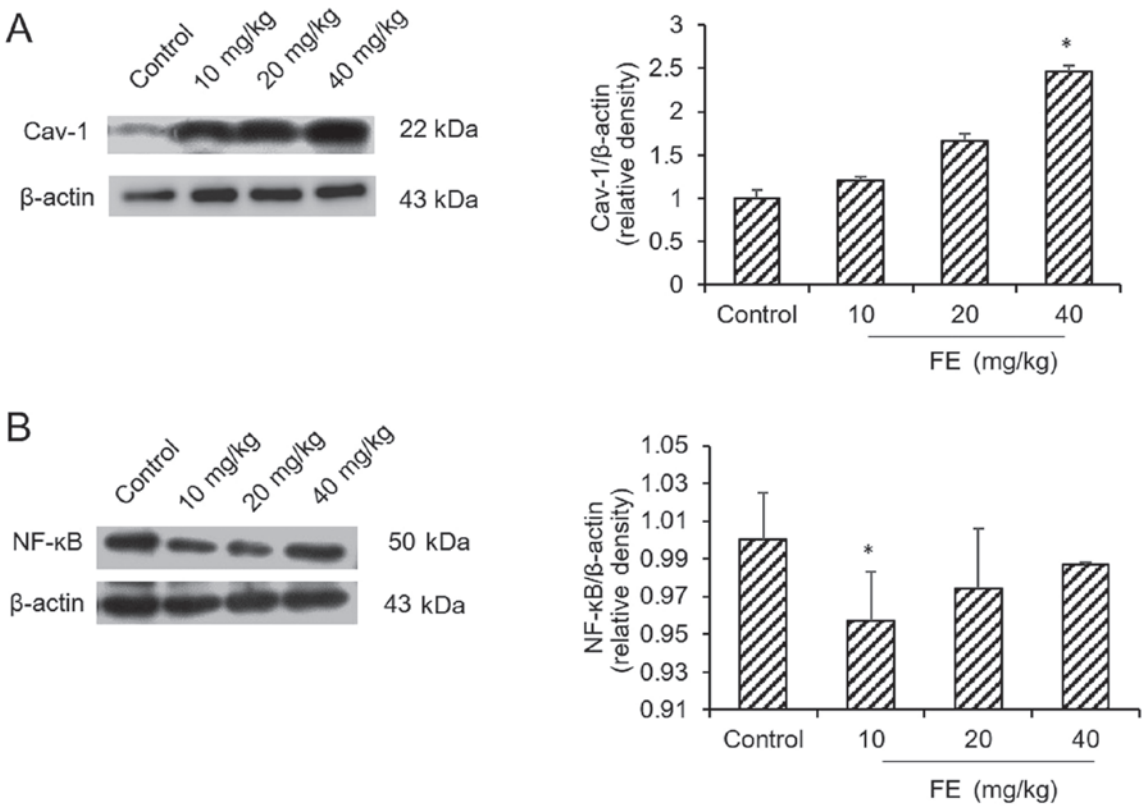

Figure 3. Effects of FE on the expression of Cav-1 and NF-κB. (A) Cav-1 levels as determined via western blotting. (B) NF-κB levels as determined via western blotting. N=3. *P<0.05 vs. Control. FE, Flor·Essence; Cav-1, caveolin-1.

Effects of FE on the expression of NF-κB and Cav-1. The results indicated that the mice supplemented with FE expressed more Cav-1 than the control mice (Fig. 3A). Additionally, it was demonstrated that the expression of NF-κB was downregulated in 10 mg/kg FE group compared with the control (Fig. 3B).

Developing a mouse model of immunosuppression. CP was administered to mice via intraperitoneal injection every other day for 8 days (Fig. 4). Immunological parameters were measured, and immune damage and immunosuppression was observed. Compared with the control group, the white blood cell count, spleen index, and IL-2 and TNF-α levels in serum were decreased significantly. This indicated that the immune function of CP group was compromised.

Effects of FE on immune cells in the mouse model of immunosuppression. The previous results obtained during the present study indicated that FE can increase the relative spleen size of mice, the phagocytic ability of peritoneal macrophages and the antitumor cytotoxicity of NK cells against K562 cells. To further investigate the effects of FE, a CP-induced model of immunosuppression was established, and the effects of treatment of these mice with FE on immune cell properties were analyzed.

The viability of spleen lymphocytes in control- or FE-treated normal mice and mice immunized with CP was detected. The results showed that the activity of spleen lymphocytes in the CP group was significantly decreased, and low (10 mg/kg) and high (40 mg/kg) concentrations of FE attenuated the effects of CP on cell viability. It was shown that FE could significantly increase the activity of spleen lymphocytes from immunosuppressed mice (Fig. 5A).

The phagocytic activity of peritoneal macrophages in normal mice and CP mice was detected. FE (10 and 40 mg/kg) was used to promote the immune activity of mice. The results showed that the phagocytic ability of macrophages was significantly increased after oral FE administration (Fig. 5B).

The killing activity of NK cells in normal mice and CP mice was detected. NK cell killing ability of mice after oral administration of FE was detected. The results showed that the ability of NK cells to kill cancer cells was significantly increased after FE administration in normal and immunosuppressed mice (Fig. 5C).

Discussion

The effects of FE on immune cell activity and cytokine production were investigated. Neutral red is recognized as a foreign substance by macrophages, and the present findings showed that FE treatment increases the phagocytic activity of macrophages isolated from the peritoneum of mice. NK cells are lymphocytes associated with autoimmune conditions that form an early immune defense during innate immunity (25). It was previously reported that the number of NK cells was reduced in patients with non-small cell lung cancer and melanoma (26). Active hexose correlated compound (AHCC) is an edible fungal extract that can affect immune responses when ingested (27). *In vivo* and *in vitro* studies have reported that the immunomodulatory effects of AHCC enhance the activity of NK, $CD8^+$ T and γδT cells (27). The present study showed that

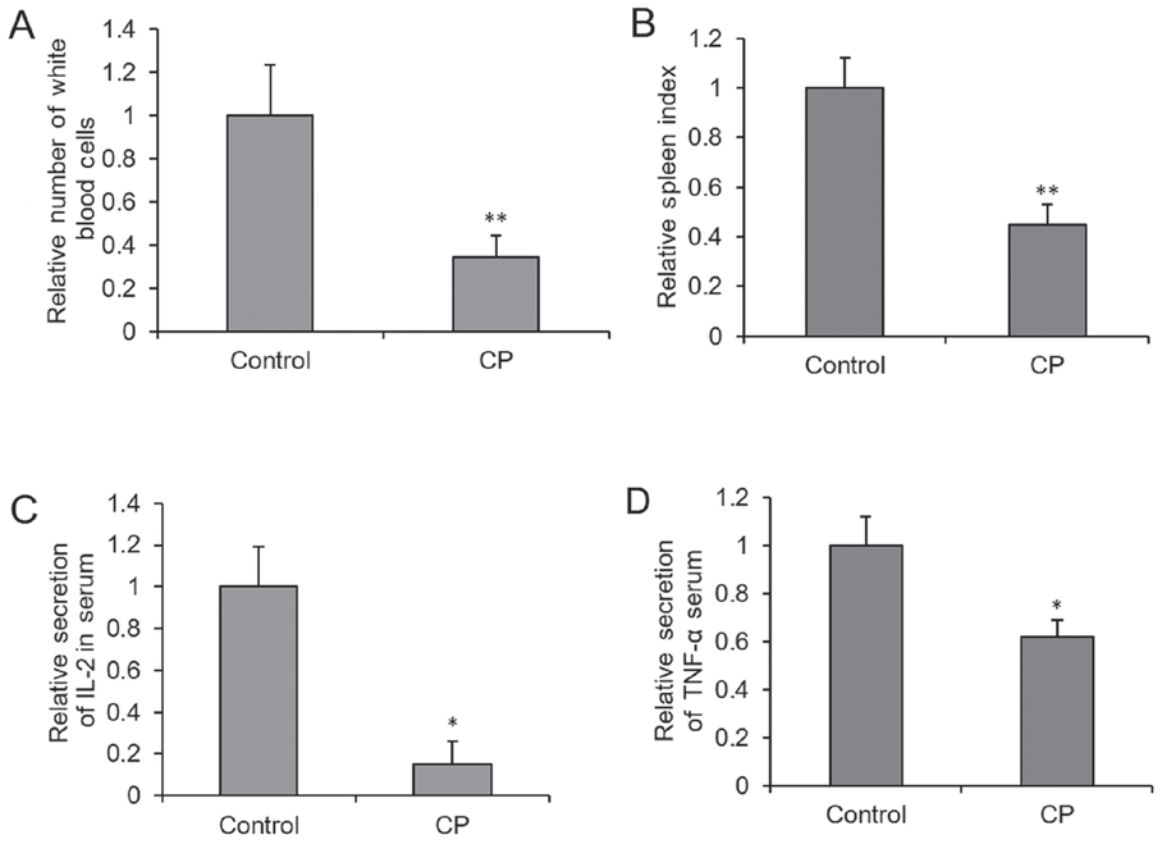

Figure 4. Developing a mouse model of immunosuppression. (A) Effects of CP on the number of white blood cells in mice. (B) Effects of CP on the spleen index of mice. (C) IL-2 protein levels were estimated by ELISA. (D) TNF-α protein levels were estimated by ELISA. *P<0.05 and **P<0.01 vs. Control. N=3. CP, cyclophosphamide; IL, interleukin; TNF, tumor necrosis factor.

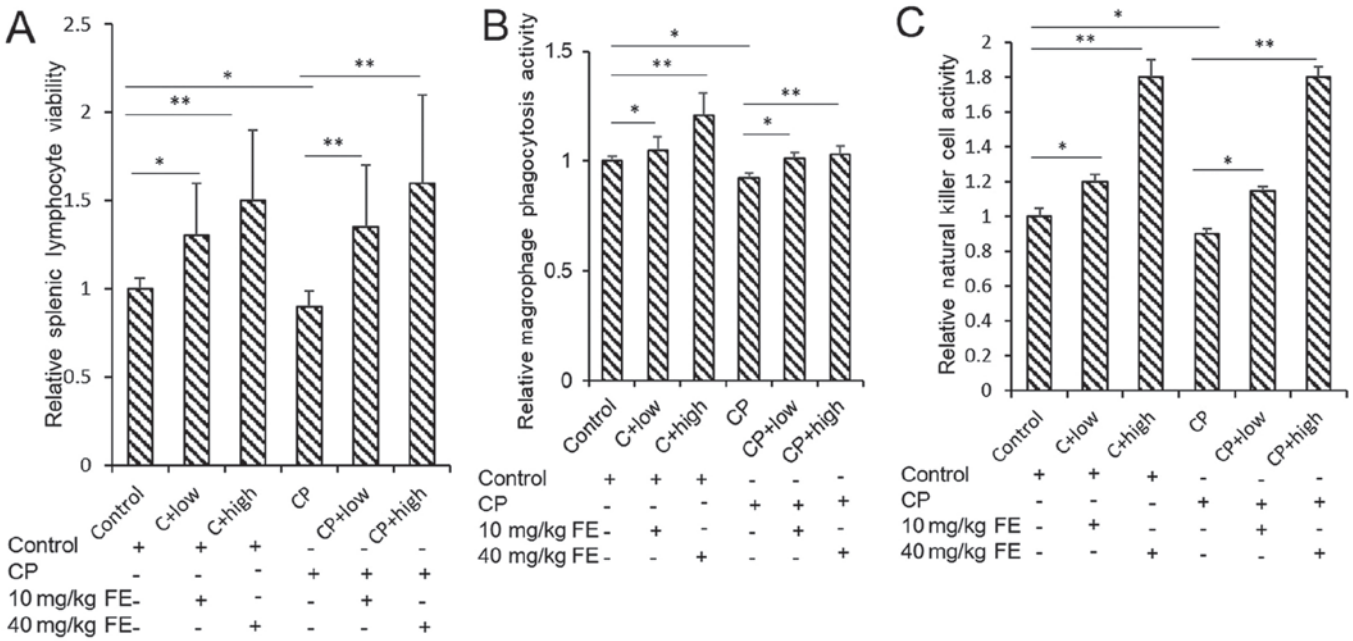

Figure 5. Effects of oral administration of FE in a mouse model of immunosuppression. (A) Effects of FE on the viability of spleen lymphocytes from immunosuppressed mice. (B) Effects of FE on the phagocytic activity of macrophages from immunosuppressed mice. (C) Effects of FE on the cytotoxic effects of natural killer cells against K562 cells. N=3. *P<0.05 and **P<0.01. FE, Flor·Essence; CP, cyclophosphamide; C, Control.

FE can enhance the killing ability of mouse NK cells, indicating that FE promotes innate immune responses. There is a strong association between NK activity and changes in spleen cells (28). In addition, FE also induced the production of IFN-α and IFN-β as determined via semi-quantitative PCR analysis. The data demonstrated that FE-supplemented mice exhibited

greater IFN-α and IFN-β mRNA expression than control mice. IFN-α and IFN-β can activate NK cells, induce antiviral states and inhibit viral republication, which are important for host defenses against viruses (29). IL-12 is a stimulating factor of NK cells, inducing the production of IFN and TNF, and activating NK cells to stimulate their differentiation, enabling NK cells to produce more TNF-α; therefore, IL-12 and TNF-α are associated with adaptive immunity (30-32). IFN-γ is an important component of the body's immune system, and is produced by activated T cells and NK cells (33). It can effectively resist viral infection and tumor formation, and promote the body's immune response (34). Therefore, IL-12 and IFN-γ levels were evaluated in peripheral blood via ELISA. Results showed that FE-supplemented mice exhibited increased secretion of IFN-γ and IL-12.

FE has previously been reported to enhance cell inflammation. Cheung *et al* (20) reported that FE inhibits NO production in a concentration-dependent manner in LPS-stimulated RAW 264.7 cells. Long *et al* (35) reported that ovarian cancer stem cells upregulate chemokine ligand 5 and activate NF-κB signaling through autocrine signaling, and induce tumor metastasis. Inhibiting the key inflammatory transcription factor NF-κB may suppress tumor invasion (36). NF-κB is associated with cancer, and is located downstream of the oncogenes Ras, Myc and Ret (37). In addition, NF-κB plays a key role in connecting inflammation with cancer (38). In the present study, it was demonstrated that expression of NF-κB was downregulated in animals supplemented with FE, suggesting that FE may dampen inflammation through NF-κB.

Cav-1, a structural protein of the cell membrane, is involved in tissue homeostasis, inflammation, oxidative stress, microbial clearance, and fibrosis (39). Cav-1 serves an important role in regulating inflammatory cell signaling in leukocytes (40). A previous study reported interactions between Cav-1 and Toll-like receptor 4, as detected by immunofluorescence in MCF-10A and MCF-10ACE cells (40). This interaction may influence the downstream regulation of inflammation-associated gene expression (41). Therefore, the expression of inflammation-related protein Cav-1 was analyzed. The results indicated that the mice supplemented with FE expressed more Cav-1 than the mice without FE, suggesting that FE may suppress inflammation via enhanced Cav-1 expression.

In the present study, increases in the proliferation of splenocytes, phagocytosis of macrophages and cytotoxicity of NK cells towards K562 cells were observed following FE treatment in a mouse model of CP-induced immunosuppression. The mechanism of CP-induced immunosuppression is similar to that of nitrogen mustard (42). CP is crosslinked with DNA to inhibit the synthesis of DNA and interfere with RNA function, inhibit tumor growth; however, when killing tumor cells, CP can damage the immune organs and inhibit humoral immunity (43). The enhancement effects of FE against immunosuppression suggested that FE may be effective as an adjuvant to chemotherapy or for use in patients with reduced immune function.

Acknowledgements

Not applicable.

Funding

This study was supported by Scientific and Technological Developing Scheme of Ji Lin Province, Liaoning Provincial Natural Science Foundation (grant no. 2015020568) and Liaoning Provincial Department of Education Research Project (grant no. L201783647)

Availability of data and materials

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

Authors' contributions

JW, JL and CQ were involved in conceiving the study, acquiring and analyzing the data, and writing the original draft. YW, YZhu, YZha and HL were involved in designing the study and analyzing the data. BZ and YS analyzed data. WZ designed the project, and acquired funding and resources. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Liaoning Normal University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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