

STUDIES ON IMMUNOSTIMULATORY EFFECT OF EFCOVIDA POWDER ON CYCLOPHOSPHAMIDE-INDUCED IMMUNOSUPPRESSION IN MICE

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To evaluate the immunostimulatory activity of Efcovida powder, we induced immunosuppression in Swiss mice by cyclophosphamide (CP). Efcovida powder at two doses of 60 mg/kg b.w and 120 mg/kg b.w were administered orally for seven consecutive days to animals. CP (200 mg/kg i.p.) was injected on the 4th day. The result showed that Efcovida powder at the dose of 60 mg/kg b.w only posed an immunostimulating potency in the cyclophosphamide-induced immunosuppression model while Efcovida powder at the dose of 120 mg/kg b.w significantly ameliorated the effects of CP on delayed-type hypersensitivity (DTH) response, leukocyte quantity, relative thymus weight, serum IL-4, IL-6 concentration, and micro-histological images of spleen and thymus. This suggested the potential of Efcovida powder to treat immunosuppression diseases in clinical practice.

Keywords: Immunostimulatory activity, immunosuppression, swiss mice, cyclophosphamide, efcovida.

I. INTRODUCTION

Immunosuppression is a state of immunity responding to pathogens below the required level.¹ Immunostimulators used with chemotherapy drugs activate or induce the mediators or components of the immune system.² Therefore, the study of new immunostimulatory agents has great promises concerning the prevention and treatment of immunosuppressive diseases.

Most prescription immunostimulants

(levamisole, isoprinosine...) are synthetic substances with serious side effects. There is a growing interest in using natural immunomodulators to modulate the complex immune system. A large number of natural materials have been suggested to have immunomodulatory effects and influence the immune system of the human body.³

Efcovida powder produced by Trinh Nang Hi-Tech Co., Ltd. contained *Endo Fullerene*, *Nano Curcumine*, *Glycyrrhiza uralensis*, *Ramulus Cinnamoni*, *Cordyceps militaris* and *Zingiber officinale*. In preclinical research, these components were proven to possess a

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significant immunostimulatory effect on both the cell-mediated and humoral immune systems.^{4,5} Moreover, polysaccharides from *Cordyceps militaris* have the tendency to overcome CY-induced immunosuppression, and significantly enhanced the function of spleen lymphocytes and macrophages.⁶ So far, no report was available about the impact of a combination product from these components on the immune system. As such we aimed to validate the immunostimulatory potency of Efcovida powder using the immunosuppression induced by cyclophosphamide in experimental animals.

II. MATERIALS AND METHODS

Preparation of Efcovida powder

The preparation of Efcovida was produced by Trinh Nang Hi-Tech Co., Ltd. in powder formulation. Each bottle of 250 mg Efcovida powder contained 44.1 mg *Endo Fullere*, 44.1 mg *Nano Curcumine*, 44.1 mg *Glycyrrhiza uralensis*, 44.1 mg *Ramulus Cinnamoni*, 44.1 mg *Cordyceps militaris*, 29 mg *Zingiber officinale* and excipients were sufficient.

Drugs and chemicals

Cyclophosphamide (Endoxan 200 mg, Baxter Oncology GmbH, Germany) was a standard immunosuppressive agent. Levamisole was obtained from Sigma (Aldrich) and used a positive control in this experiment. Sheep red blood cells (SRBC) and OA solution (ovalbumin + Al(OH)₃) were used as the antigenic materials.

Animals

Swiss albino mice of either sex, weighing 20 ± 2 g were purchased from the National Institute of Hygiene and Epidemiology. The mice were kept in cages (10 animals per cage) and provided with water and standard food ad libitum and were adapted to their laboratory condition within seven days.

Experimental design

The animals were divided randomly into five groups of ten mice per cage. Group I (control) was given an i.p. injection of physiological saline. Group II (model) was injected CP with a single dose (200 mg/kg) on the 4th day of the experiment. Group III (levamisole 10 mg/kg + CP), group IV (Efcovida powder at the dose of 60 mg/kg b.w (equivalent to the dose of 250 mg/day/person, conversion ratio 12) + CP)) and group V (Efcovida powder at the dose of 120 mg/kg b.w (equivalent to the dose of 500 mg/day/person, conversion ratio 12) + CP)). Mice were administered levamisole and Efcovida powder orally for seven consecutive days, with a single injection of CP on the 4th day. Efcovida powder was dissolved with distilled water before giving orally for mice once daily. On the 8th day, mice were sacrificed to collect blood samples, spleen and thymus to evaluate immune parameters. All indexes including delayed type hypersensitivity (DTH) response, leukocyte quantity, relative organ weight, serum IL-4, IL-6, IFN- γ , TNF- α and IgG were performed at the Pharmacology Department of Hanoi Medical University.

Delayed type hypersensitivity (DTH) response

On the 2nd day of the model, animals were given an i.p. injection of sheep red blood cells and injected OA 0,1 mL into the spinal cord. DTH reaction was elicited five days later by injecting of OA 50 μ L into right hind paw and physiological saline into the left after measuring the footpad's thickness. After 24 hours, the paw volume was measured again to assess the swelling degree of the footpad.

Counting of leukocyte quantity

Blood samples were collected on the day of sacrifice to determine the number of total WBC, lymphocytes, neutrophils and monocytes by using an automatic blood analyzer.

Relative organ weight

The related weight of the spleen and thymus was calculated using the following formula:

$$\text{Relative organ weight} = \frac{\text{Organ weight (mg)}}{\text{Body weight (g)}}$$

Histopathological study of spleen and thymus

Micro-histological examination of the spleen and thymus was carried out at the Department of Pathology, Military Hospital 103. Nguyen Thanh Chung, MSc, MD, gave results of pathological image analysis.

Assay for serum IL-4, IL-6, IFN-g, TNF- α and IgG

On the 8th day of the experiment, blood samples were assayed to measure the concentration of serum IL-4, IL-6, IFN-g, TNF- α and IgG using an ELISA kit (Cloud-Clone Corp., USA) according to the manufacturer's protocols. All blood samples was centrifuged at 1000 rpm and 2-8°C for 15 minutes in order to collect the plasma. The plasma was prepared step-by-step following the instruction. At the end of the assay procedure, samples were measured at 450 nm by using the microplate reader to calculate

concentrations of these cytokines.

Statistical analysis

The data were expressed as the Mean \pm SD and statistical analysis was carried out employing a student's t-test.

III. RESULTS

1. Effect of *Efcovida* powder on delayed-type hypersensitivity (DTH) response

Administration of CP (200 mg/kg, i.p) showed a significant decrease in the DTH response as compared with the control group (group I) ($p < 0.05$). There was a significant growth in the paw volume in groups treated with levamisole ($p < 0.05$) as compared with group II. In the group treated with *Efcovida* at the dose of 60 mg/kg/day (group IV), no significant difference was observed in the DTH response as compared with the model group ($p > 0.05$). There was an upward trend in the DTH response in the group treated with *Efcovida* at the dose of 120 mg/kg/day as compared with the model group, but no significant difference was observed ($p > 0.05$) (Figure 1).

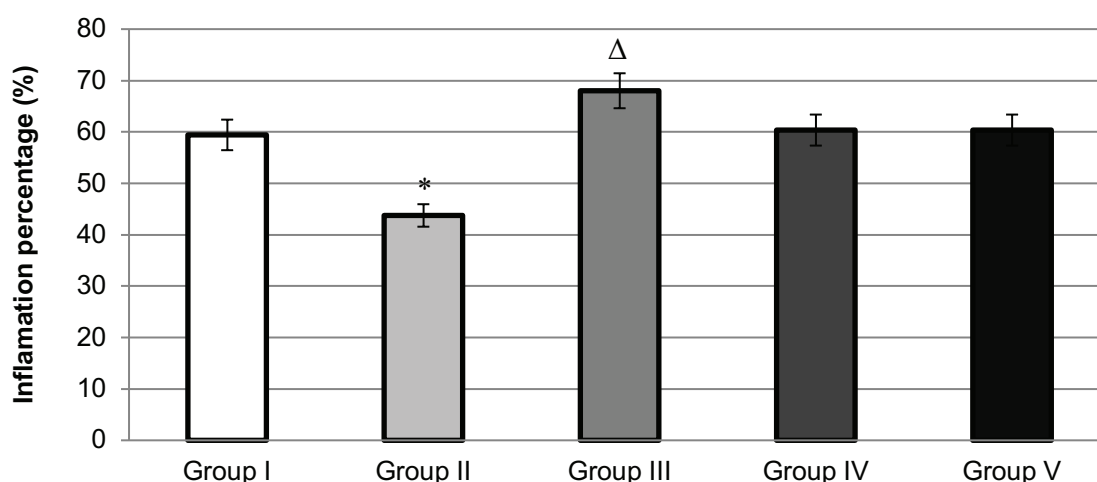


Figure 1. Effect of *Efcovida* on delayed-type hypersensitivity (DTH) response

* compared with group I ($p < 0.05$).

Δ Groups III was compared with group II ($p < 0.05$).

2. Effect of Efcovida powder on leukocyte quantity

Mice treated with CP showed a significant reduction in leukocyte quantity as compared with the control group. Levamisole increased the white blood cells (WBC) slightly, lymphocytes (LYM), neutrophils (NEU) and monocytes (MONO) as compared with group II. In groups

treated with Efcovida at the doses of 60 mg/kg/day and 120 mg/kg/day, no significant difference was observed in total WBC count, lymphocytes, neutrophils and monocytes count as compared with the model group ($p > 0.05$)(Table 1).

Table 1. Effect of Efcovida powder on leukocyte quantity

Group	Treatments	Leukocyte quantity (Means \pm SD)			
		WBC (G/L)	LYM (G/L)	NEU (G/L)	MONO (G/L)
I	Control	5.84 \pm 1.02	3.25 \pm 0.78	1.39 \pm 0.44	1.20 \pm 0.50
II	Model	0.93 \pm 0.39 ***	0.62 \pm 0.27 ***	0.25 \pm 0.11 ***	0.06 \pm 0.05 ***
III	Levamisole 10 mg/kg + CP	1.11 \pm 0.68 ***	0.64 \pm 0.26 ***	0.30 \pm 0.21 **	0.17 \pm 0.29 ***
IV	Efcovida powder 60 mg/kg + CP	0.90 \pm 0.43 ***	0.58 \pm 0.26 ***	0.24 \pm 0.18 ***	0.08 \pm 0.15 ***
V	Efcovida powder 120 mg/kg + CP	0.89 \pm 0.38 ***	0.54 \pm 0.27 ***	0.23 \pm 0.13 ***	0.12 \pm 0.18 ***

** ,*** compared with group I ($p < 0.01$, $p < 0,001$).

3. Effect of Efcovida powder on relative organ weight

Figure 2 showed that the relative spleen weight of the animals treated with Efcovida powder at a 60 mg/kg dose increased slightly compared to group II but no significant action

was observed ($p > 0.05$). Besides, there was an upward trend in thymus relative weight in mice treated with Efcovida powder at the dose of 120 mg/kg as compared with group II ($p > 0.05$).

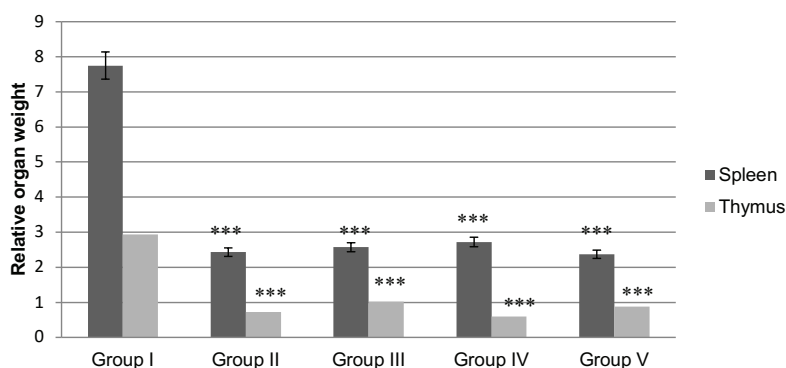


Figure 2. Effect of Efcovida powder on relative organ weight

*** compared with group I ($p < 0.001$).

4. Effect of Efcovida powder on serum antibody, IL-4, IL-6, IFN-g and TNF-α concentration

Table 2 below illustrated that there was a slight increase in serum IL-4, IL-6 and TNF-α concentration in the group treated with levamisole as compared to group II. Efcovida at the dose of 60 mg/kg showed an upward trend in serum IL-4 and IL-6 concentration and a significant increase in TNF-α concentration compared to group II. In

the group treated with Efcovida at the dose of 120 mg/kg, there was a significant increase in serum IL-4 concentration and a slight increase in serum IL-6 concentration compared to group II. There was no substantial difference between groups treated with Efcovida and group II in IgG concentration ($p > 0.05$).

Table 2. Effect of Efcovida powder on serum antibody, IL-4, IL-6, IFN-g and TNF-α concentration

Group	IgG concentration Mean ± SD (µg/mL)	IL-4 concentration Mean ± SD (pg/mL)	IL-6 concentration Mean ± SD (pg/mL)	TNF-α concentration Mean ± SD (pg/mL)	IFN-g concentration Mean ± SD (pg/mL)
I	203.51 ± 20.51	9.94 ± 3.06	8.64 ± 3.62	20.11 ± 7.90	502.35 ± 108.28
II	89.47 ± 35.89***	5.13 ± 2.73**	5.75 ± 2.62	14.10 ± 4.48	367.46 ± 138.52*
III	74.34 ± 32.37***	6.09 ± 2.69**	7.86 ± 3.38	14.99 ± 9.90	343.93 ± 141.27*
IV	78.79 ± 32.59***	7.71 ± 2.69	7.25 ± 2.92	20.82 ± 7.24 ^Δ	345.80 ± 136.19*
V	66.58 ± 35.80***	8.14 ± 2.74 ^Δ	8.23 ± 3.18	14.04 ± 7.97	356.65 ± 167.40*

*, **, *** compared with group I ($p < 0.05$, $p < 0.01$ and $p < 0.001$).

^Δ Groups IV, V were compared with group II ($p < 0.05$).

5. Histopathological study of spleen and thymus

The micro-histological images (Figure 3 and 4) demonstrated that in the spleen and thymus of animals treated with CP, the number of lymphocytes decreased dramatically compared

to the control mice. Levamisole and Efcovida powder at both doses significantly restored the lymphocyte quantity in the spleen and thymus.

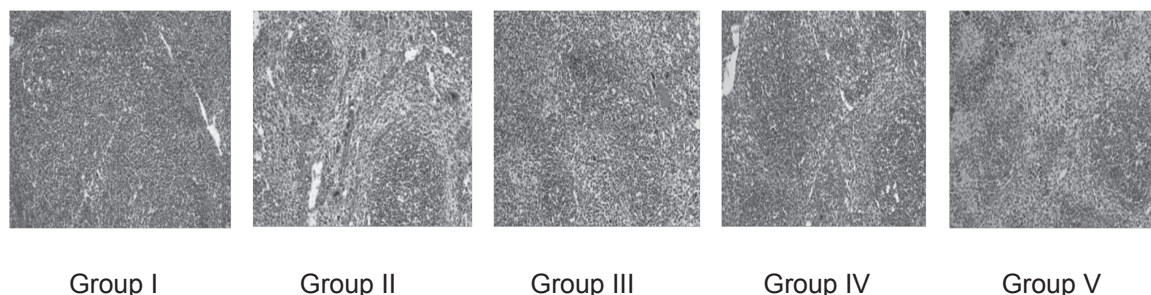


Figure 3. Micro-histopathological images of spleens (HE × 20)

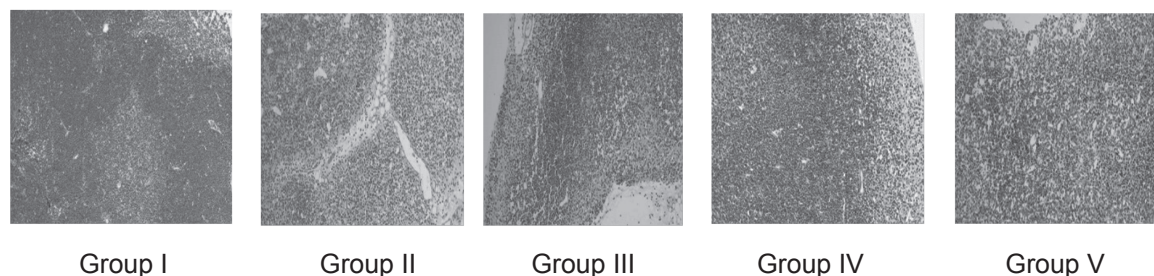


Figure 4. Micro-histopathological images of thymuses (HE × 20)

IV. DISCUSSION

Cyclophosphamide is one of the most commonly used alkylating agents which produces toxic side effects including immunotoxicity, hematotoxicity and mutagenicity. It has a pronounced effect on lymphocytes and is usually used as an immunosuppressant. In this study, CP at the dose of 200 mg/kg i.p caused a significant decrease in some parameters including: delayed-type hypersensitivity (DTH) response; leukocyte quantity; relative organ weight; serum antibody, IL-4, IL-6, IFN-g and TNF- α concentration.

Bone marrow is a site of continued proliferation and turnover of blood cells, and is a source of cells involved in immune reactivity. T-lymphocytes and other key immune system cells are known to activate the production of antibodies and polymorphonuclear granulocytes to destroy the antigen. Loss of stem cells and the inability of bone marrow to regenerate new blood cells will result in leucopenia. In this study, no significant change was observed in total WBC count, lymphocytes, neutrophils, and monocytes count in groups treated with Efcovida compared with the model group.

In the DTH test, the DTH response directly correlates with the mediated immunity was considerable in animals treated with levamisole and Efcovida powder. This explains that after challenged by antigen, the

sensitized T-lymphocytes were converted to lymphoblasts, secreting various molecules, including proinflammatory cytokines, attracting scavenger cells to the reaction site. An increase in foodpad thickness indicated the stimulatory effect of Efcovida powder on the lymphocytes and accessory cell types required to express this reaction.

Cytokines secreted by many cells, including lymphocytes, have an essential role in the body's inflammation response, especially IL-4 and TNF- α . In this study, there was a significant improvement in TNF- α concentration in mice treated with Efcovida at the dose of 60 mg/kg and IL-4 concentration in mice treated with Efcovida at the dose of 120 mg/kg as compared to the model group.

Antibody production induced by thymus-dependent antigen (sheep red blood cells) requires the co-operation of T- and B-lymphocytes and macrophages. The high value of the IgG titer in mice treated with Efcovida powder expressed the immunostimulation of Efcovida powder through humoral immunity. However, no substantial difference was observed between groups treated with Efcovida and group II in IgG concentration ($p > 0.05$).

There was an upward trend in the relative spleen weight of group IV and the relative thymus weight of group V as compared to the

model group. The increase in thymus and spleen weight was accompanied by the increase in cell numbers, improving the stimulatory effect of this powder on the lymphocytes and bone marrow haematopoietic cells. This result was consistent with the histopathological improvement in the thymus and spleen in experimental groups treated with Efcovida (Figure 3 and 4).

As compared with Efcovida powder at dose of 60 mg/kg b.w, Efcovida powder at the dose of 120 mg/kg b.w. tended to improve immune indexes, but no significant difference was observed. These results indicated that Efcovida powder posed an immunostimulatory effect on cyclophosphamide-induced immunosuppression and showed that Efcovida powder could potentially be developed as a new traditional herbal medicine. Our study was consistent with the results from the previous report about the toxicity of components in Efcovida powder. According to Shin S (2010), *Cordyceps militaris* increased proinflammatory cytokines through the activation of NF- κ B, further suggesting that CME may prove useful as an immune-enhancing agent in the treatment of immunological disease. Nanoparticulate curcumin stimulated higher early cell-mediated immune response and humoral immune response compared to the free curcumin through delayed-type hypersensitivity assays, white blood cells, weight of the lymphoid organs and antibody titre.

V. CONCLUSION

Efcovida powder posed stimulatory impacts on the immune system suppressed by CP. Oral administration of Efcovida powder at a dose of 60 mg/kg b.w (equivalent to the dose of 250 mg/day/person) for seven consecutive days had an immunostimulating potency in the cyclophosphamide-induced immunosuppression model through indexes including relative spleen weight, micro-

histological images of spleen and thymus, serum IL-4, IL-6 and TNF- α concentration. Efcovida at the dose of 120 mg/kg/day (equivalent to the dose of 500 mg/day/person) significantly improved the effects of CP on relative thymus weight, micro-histological images of spleen and thymus, delayed-type hypersensitivity (DTH) response, serum IL-4 and IL-6 concentration. There was an improvement in immune indexes of group treated with Efcovida powder at the dose of 120 mg/kg b.w. compared to Efcovida powder at the dose of 60 mg/kg b.w., but no significant difference was observed.

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