IMMUNOENHANCEMENT EFFECTS OF THE HERBAL FORMULA ANTI-U200 ON CYCLOPHOSPHAMIDE-INDUCED IMMUNOSUPPRESSION IN MICE

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Received date: 09/09/2024

Reviewed date: 30/09/2024

Accepted date: 30/09/2024

ABSTRACT

Anti-U200 is an herbal blend comprising Ganoderma lucidum, Hedyotis diffusa, and Scutellaria barbata is known to have immunomodulatory effects. ***Objectives:*** *We examined the immuno-enhancing effect of this herbal blend on cyclophosphamide (CYP)-induced immunosuppression.* ***Methods:*** *Swiss mice were assigned to one of five groups: the intact control and four CYP treatment groups (one control, one reference (levamisole), and two with the application of Anti-U200 at different dosages; 1.92 or 5.76 capsules/kg). Mice received one of the experimental treatments after being injected with CYP to induce myelosuppression and immunosuppression.* ***Results:*** *Anti-U200 therapy improved lymphoid organ atrophy in histological assessments and ameliorated the CYP-induced decreases in spleen and thymus weights in immunosuppressed animals. It also led to beneficial changes in leucocyte counts and boosted TNF-α and IgM serum levels.* ***Conclusions:*** *We have proven that Anti-U200, a combination of three immunomodulatory herbs, is an efficient immunomodulatory agent that can strengthen the immune system.*

Keywords*: Anti-U200, cyclophosphamide, immunomodulatory, mixed herbal, mice.*

1. INTRODUCTION

Nowadays, one of the main concerns with chemotherapy is the adverse effects connected to the delivery of chemotherapeutic medicine [1]. Myelosuppression and immunosuppression are brought on by both cytotoxic and immunomodulatory chemotherapy, which impair bodily processes and lower quality of life [2]. Chemotherapy for an extended period may also lead to immunodeficiency, which increases vulnerability to infections and lowers immunosurveillance against cancer [3]. Fever, flu-like symptoms, and allergic responses are among the known side effects of certain immunomodulatory medications, even though they can be used to avoid these side effects [4]. Natural herbal remedies, on the other hand, are thought to be potentially useful immunomodulators with negligible or no side effects.

Anti-U200 is a herbal blend that consists of three medicinal plants containing dry extracts from *Scutellaria barbata*, *Hedyotis diffusa*, and *Ganoderma lucidum*. Anti-tumor and immunomodulatory effects have been demonstrated for herb constituents in the Anti-U200 preparation [5-7]. Nevertheless, there is a lack of study on the impacts of these immunomodulatory herbal mixtures on immunological modulation, despite the assumption that herbal formulations are more synergistic than individual immunomodulatory herbal components. Therefore, it is anticipated that the combination of these herbs in Anti-U200 would have a more pronounced immunomodulatory effect. In this work, we investigated the immune-enhancing

effects of Anti-U200 on cyclophosphamide-induced immunosuppression in mice, which have been employed as a useful animal model for assessing the immunomodulatory effects of natural substances.

2. MATERIALS AND METHODS

2.1. Chemicals

Anti-U200 was supplied by the Institute of Traditional Medicine Research and Development (Hanoi, Vietnam). This product comprises a 93% herbal blend (*Scutellaria barbata*, *Hedyotis diffusa*, and *Ganoderma lucidum*). Other constituents include magnesium carbonate, 7% PVP K30/ethanol 96%, aerosil, and talc. The lowest dose of Anti-U200 used in this study was 1.92 capsules/kg, which was calculated by multiplying the recommended dosage for humans (8 capsules per day) by the conversion rate of animal doses to human-equivalent doses (12-fold), and we also assessed the effects of 5.76 capsules/kg, representing three-fold increases in the administered dosage.

Cyclophosphamide (Endoxan 200 mg, Baxter Oncology GmbH, Germany) was used as the immunosuppressive agent. Levamisole was obtained from Sigma (Aldrich) Chemicals Pvt. Ltd. USA and used as a positive control in this experiment. Sheep red blood cells (SRBC) and OA solution (ovalbumin + $Al(OH)_3$) were used as the delayed-type hypersensitivity inducer. Cytokine ELISA kits and antibodies were purchased from Cloud-Clone Corp (CCC, USA).

2.2. Experimental animals

50 mice of either sex (20–30 g; 2-3 months old) were used. The animals were supplied by the National Institute of Hygiene and Epidemiology (Hanoi, Vietnam). The acclimatization of test animals was carried out by placing mice in metal cages with standard environmental conditions for 7–14 days at room temperature and with adequate ventilation before the experiment. The animals were provided with a standard pellet diet and water ad libitum.

2.3. Methods

The experiment was carried out following our previous publication [8].

After being adapted to the environment for 7-14 days, these mice were randomly divided into 5 groups consisting of 10 mice each. Mice were treated as Table 1. The groups were the non-CTX group (Normal), immunosuppressive model group (CYP), positive control group (100 mg kg^{-1} levamisole), 1.92 capsules kg^{-1} Anti-U200 group, and 5.76 capsules kg^{-1} Anti-U200 group. Except for the normal group, the other groups were injected 200 mg kg^{-1} CYP on the 4th day to establish the immunosuppressive mice model. In the Anti-U200 experimental group, mice were given different doses of Anti-U200 by gavage for 7 days. On the 8th day, mice were anesthetized by chloral hydrate (250 mg kg^{-1} i.p.) and then euthanized to collect blood samples, spleen, and thymus to evaluate immune parameters.

Table 1. Mouse treatment schedule.

Group	Treatment	Duration
Normal	Distilled water (0,2 mL/10 g bw, gavage)	7 days
CYP	200 mg kg^{-1} CYP (inject)	Day 4
Levamisole	100 mg kg^{-1} Levamisole (gavage) 200 mg kg^{-1} CYP (inject)	7 days Day 4
Low-dose Anti-U200	1.92 capsules kg^{-1} Anti-U200 (gavage) 200 mg kg^{-1} CYP (inject)	7 days Day 4
High-dose Anti-U200	5.76 capsules kg^{-1} Anti-U200 (gavage) 200 mg kg^{-1} CYP (inject)	7 days Day 4

Determination of thymus and spleen indices

Mice were sacrificed 24 hours after the last intragastric administration, and then each mouse's thymus and spleen were aseptically removed and weighed. The organ index was calculated as follows:

$$Index\ (‰) = \frac{weight\ of\ spleen\ or\ thymus\ (mg)}{body\ weight\ (g)}$$

Leukocyte Counts

On the day of sacrifice, blood samples were collected from carotid arteries to determine the total WBC, lymphocytes, neutrophils, and monocytes using a HORIBA® ABX MircoES60 blood analyzer.

Determination of Cytokines in Serum by ELISA

Serum samples were prepared by centrifuging the whole blood at 3500 rpm at 4^0C for 10 min. The levels of immunoglobulin (IgM) and TNF-α in the serum were measured according to the ELISA kit instructions.

Delayed type hypersensitivity (DTH) test

On the second day of the experiment, animals were given an i.p. injection of 0.5 ml of 5% (v/v) sheep red blood cells and injected subcutaneously OVA 0,1 mL into the nape of the neck site. DTH reaction was elicited 5 d later by the injection of OVA 50 µL into right the hind paw and physiological saline into the left one after measuring the thickness of the footpad in the center of walking pads using an INSIZE® digital external caliper (Code: 2312–20). After 24 h, the paw volume was measured again to assess the swelling degree through differential percentage between the two footpads.

Histopathological analysis

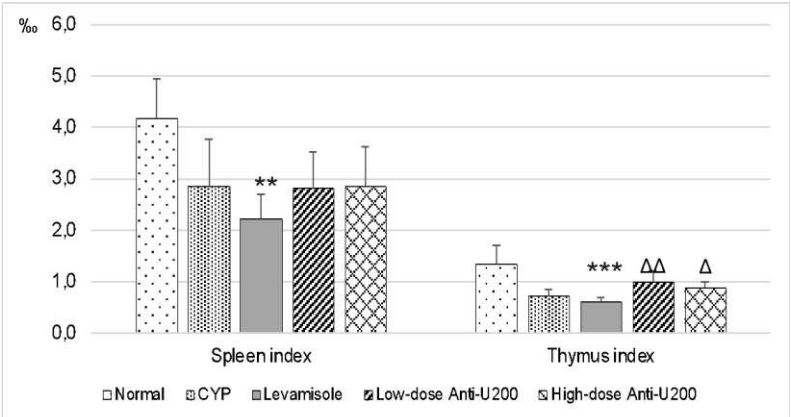
A histopathological examination was performed in a total of 15 spleens/thymi: 3 in each group. After their removal, the organs were weighed using a digital scale (Precisa®, Swiss, Model 321 LX Type 2200 C), and then preserved in 10% formalin for fixation. Sections stained with hematoxylin and eosin and examined under a light microscope. The images were photographed digitally at 100x.

2.4. Statistical analysis

Data are shown as mean ± SD. The statistical significance of the differences between various groups was determined by Student's t-test using Microsoft Excel version 2010. Values of $p < 0.05$ were considered statistically significant.

3. RESULTS

3.1. Effects of Anti-U200 on organ indices



***p<0.01, ***p<0.001 vs. Normal group; $^{\Delta}$p<0.05, $^{\Delta\Delta}$p<0.01 vs. CYP alone.*

Figure 1. Effect of Anti-U200 on immune organ indices

The spleen and thymus indices of the normal and immunosuppressive mice are depicted in Figure 1. Compared with the normal group, the spleen and thymus

indices of the CYP-exposed group were significantly decreased. The relative thymus weights were not affected by Anti-U200 capsules. However, there was an increase in relative spleen weight in mice treated with test capsules, which was obvious in both tested doses.

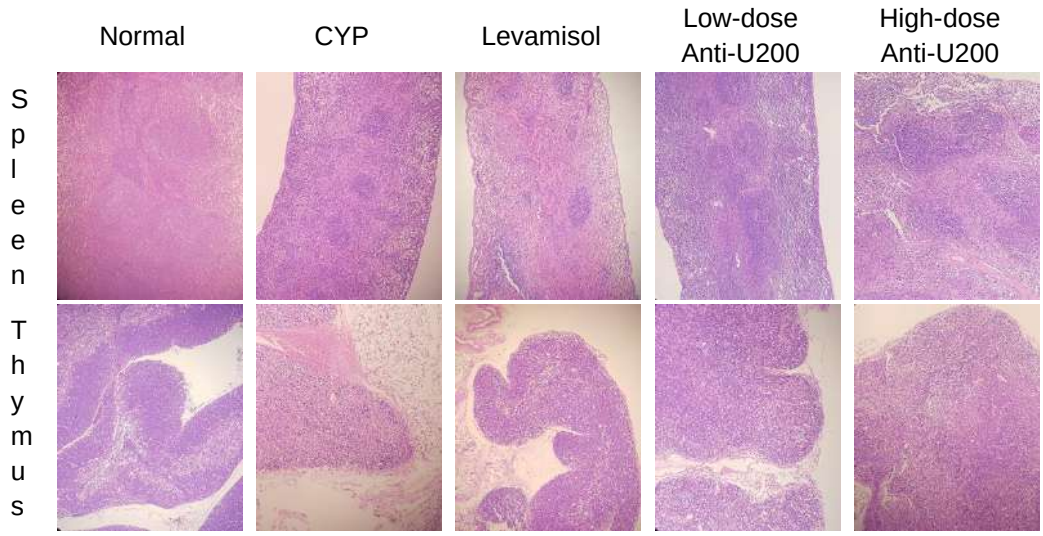


Figure 2. Representative histopathological images of the spleen and thymus

In the present study, the histomorphology of the spleen and thymus by HE staining was examined with an optical microscope (Figure 2). In the spleen, white pulp atrophy was easily observed in the model group, and red pulp and white pulp were intermixed as well when compared with those in the normal group. In the thymus, the clear tissue structures including cortex, medulla, and thymic corpuscle were observed in the normal group, which were much destroyed in the model group. In the model group, we also observed a decrease in the number of splenocytes and thymocytes. Anti-U200 administration significantly inhibited the pathological alterations in the mouse spleen and thymus induced by CYP. Besides, as the positive control, the pathological alteration of the levamisole group was also much rescued, similar to those in the Anti-U200 groups.

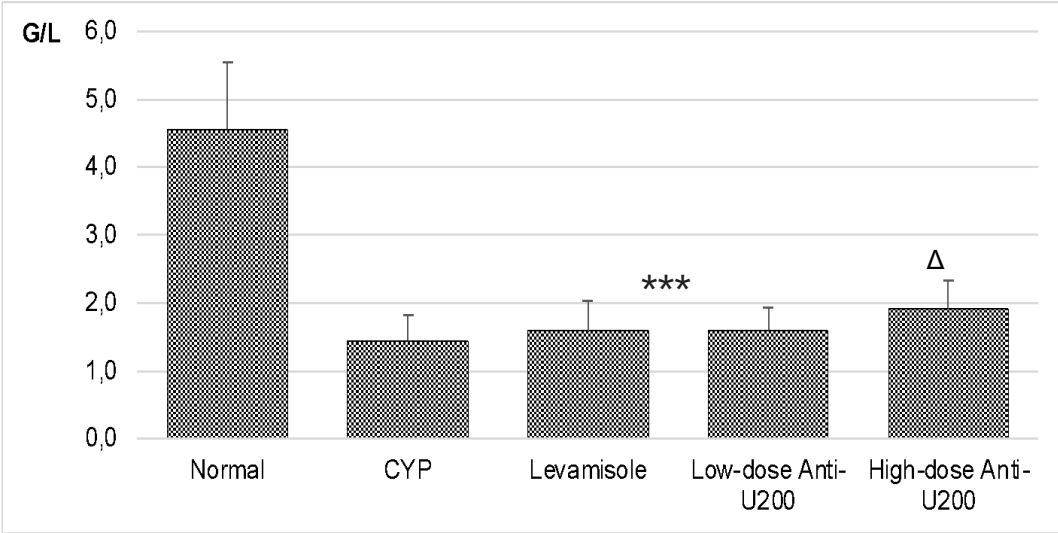
3.2. Effect of Anti-U200 on leukocyte counts

As shown in Table 2 and Figure 3, compared with the normal group, the number of peripheral blood WBCs was significantly decreased in the CYP group. When treated with Anti-U200, the number of peripheral blood WBCs tended to increase, especially in the 5.76 capsules/kg group.

Table 2. Effects of Anti-U200 on the absolute number of peripheral white blood cells

Groups	Lymphocyte (cells/mm^3)	Neutrophil (cells/mm^3)	Monocyte (cells/mm^3)
Normal	2140 ± 610	1840 ± 520	580 ± 170
CYP	700 ± 210***	580 ± 170***	170 ± 50***
Levamisol	720 ± 190	620 ± 200	240 ± 70$^{\Delta}$
Low-dose Anti-U200	940 ± 230$^{\Delta}$	500 ± 100	160 ± 50
High-dose Anti-U200	710 ± 130	930 ± 230$^{\Delta\Delta}$	270 ± 80$^{\Delta\Delta}$

****p<0.001 vs. Normal group; $^{\Delta}$p<0.05, $^{\Delta\Delta}$p<0.01 vs. CYP alone.*



****p<0.001 vs. Normal group*; $^{\Delta}$*p<0.05 vs. CYP alone.*

Figure 3. Effects of Anti-U200 on WBCs

3.3. Effect of Anti-U200 on Delayed-Type Hypersensitivity (DTH) Response

Administration of CYP (200 mg kg−1, i.p) showed a decrease in the DTH response (Table 3). There was a tendency to increase the paw volume in the group treated with Anti-U200 at two studied doses, but there was no significant difference.

Table 3. Effect of Anti-U200 on the swelling degree of footpads

Group	Swelling of foot-pads (%)
Normal	14.36 ± 2.99
CYP	7.74 ± 1,95***
Levamisol	8.32 ± 1.80
Low-dose Anti-U200	10.05 ± 3.07
High-dose Anti-U200	7.90 ± 1.75

****p<0.001 vs. Normal group.*

3.4. Effect of Anti-U200 on serum IgM and TNF-α concentrations

Table 4. Effect of Anti-U200 on serum IgM and TNF-α concentrations

Groups	TNF-α (pg/mL)	IgM (ng/mL)
Normal	30.89 ± 7.08	408638.88 ± 79384.54
CYP	17.10 ± 4.15***	100721.81 ± 19799.21***
Levamisol	25.32 ± 7.10$^{\Delta\Delta}$	127198.58 ± 24118.03$^{\Delta}$
Low-dose Anti-U200	21.63 ± 3.88$^{\Delta}$	103012.33 ± 26255.44
High-dose Anti-U200	28.04 ± 7.19$^{\Delta\Delta}$	108668.64 ± 21020.91

****p<0,001 vs. Normal group;* $^{\Delta}$*p<0.05,* $^{\Delta\Delta}$*p<0.01 vs. CYP alone.*

CYP injection caused a significant reduction of cytokine TNF-α levels in serum. Similarly, we found that the concentrations of IgM in serum were noticeably decreased in the model group when compared with that in the normal control group. As shown

in Table 4, Anti-U200 improved the CYP-induced reduction in the cytokine TNF-α and immunoglobulin (IgM) in the sera, in which, statistically significant differences were observed in TNF-α concentrations increased.

4. DISCUSSION

The immune system plays a role in protecting the body against foreign pathogens and infections, thus playing a role in the body's homeostasis. Regulation of the immune system by stimulation or inhibition helps maintain a disease-free state in an individual [9]. Therefore, immunomodulators have been widely used in medicine to control diseases. This study was conducted to evaluate the immunomodulatory activity of Anti-U200 by evaluating the effects of the test capsules on WBC and differential counts, spleen and thymus weight, delayed-type hypersensitivity response, TNFα, and IgM levels. Immunodeficiency in experimental animals was induced by intraperitoneal injection of a single dose of 200 mg/kg of cyclophosphamide, a potent immunosuppressive agent that can suppress both humoral and cell-mediated immune responses [10].

Monitoring the weight of the spleen and thymus can indirectly assess the immune system's ability to respond to protect the body against antigen invasion. The thymus is the main place where T lymphocytes are concentrated, while the spleen is mainly B lymphocytes [9]. A single intraperitoneal injection of CYP at a dose of 200 mg/kg significantly reduced the relative weight of these two organs, corresponding to the microscopic image of a severe reduction in the size of the white pulp of the spleen and the number of lymphocytes in the thymus, which clearly shows the suppressing effect of CYP on both humoral and cell-mediated immunity. Comparing the relative thymus weight in the Anti-U200 groups with the model group, it can be seen that the test capsules significantly increased the weight of the thymus compared to the model group. There was no change in the relative spleen weight in all Anti-U200 groups compared to the model group (Figure 1). However, microscopic images of the spleen and thymus showed a partial structural improvement of both lymphoid organs in the presence of Anti-U200, specifically an increase in the size of the white pulp of the spleen and the number of lymphocytes of the thymus in the Anti-U200 groups compared to the model group (Figure 2), in other words, the mixed herbal preparation demonstrated initially a stimulating effect on cell proliferation in these lymphoid organs. Corresponding to the efficacy of Anti-U200 on increasing the weight of the above lymphoid organs was a differential increase in the concentration of TNFα ($p < 0.05$), a cytokine representing a cell-mediated immune response originating mainly from T lymphocytes concentrated principally in the thymus, and a tendency to increase the concentration of IgM antibodies ($p > 0.05$) representing humoral immunity originating primarily from B lymphocytes condensed mainly in the spleen, when compared to the model group.

The above-denoted research results suggested that Anti-U200 at doses of 1.92 capsules/kg/day and 5.76 capsules/kg/day treated orally continuously for 7 days exhibited an immune-stimulating effect on CYP-induced immunosuppressive mice by increasing the relative thymus weight, WBCs, TNFα concentration in serum, and at the same time improve microscopic images of spleen and thymus compared to the model group.

Polyherbal formulations are created by combining extracts from different medicinal plants to provide synergistic effects or to heighten desired effects. The modulation of humoral and cellular-mediated immunity in the CYP-induced immunosuppressive model by combined extracts of *Ganoderma lucidum*, *Hedyotis*

diffusa, and *Scutellaria barbata* may suggest a positive interaction between the bioactive components of the three plants, leading to the stimulatory effect. The immunostimulatory effect of combined extracts of *Scutellaria barbata*, *Hedyotis diffusa*, and *Ganoderma lucidum* indicates their potential to be developed into a promising nutraceutical for the modulation of immune responses for the prevention and treatment of various diseases, such as immunodeficiency and cancer. The side effects of biological agent immunotherapy in cancer have been documented in several studies [11]. As a result, there is a tremendous deal of potential to develop the combined plant extracts into a safe and efficient cancer treatment and prevention agent. However, more research is required to explore the molecular mechanisms underlying the synergistic effects to elucidate the immunostimulatory effect of the combined extracts.

5. CONCLUSIONS

This study concluded that polyherbal formulation Anti-U200 exhibited immunomodulatory effects in CYP-induced immunosuppressed mice by improving the relative weight of lymphoid tissues likewise the levels of WBCs and differential. In addition, the extract demonstrated a noteworthy increase in the levels of TNF-alpha and IgM in CYP-injected mice. These findings indicated the potential use of the mixed herbal extract capsule as an alternative immunomodulatory agent in the management of diseases related to immune deficiency.

Acknowledgments

The authors would like to thank all researchers who participated in supporting this study from the Department of Pharmacology, Hanoi Medical University, and the Center for Research and Early Detection of Cancer (CREDCA), Tri Duc Hospital.

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