Immune enhancement effect of an herb complex extract through the activation of natural killer cells and the regulation of cytokine levels in a cyclophosphamide–induced immunosuppression rat model

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1. Introduction

Immune system enhancement is an area of increasing interest.

Natural products are being used as alternative therapy for health care and medicinal purposes. Over the past decade, studies have recognized several important aspects of immune systems to protect the body against infection[1]. Emergence of HIV/AIDS, severe acute respiratory syndrome, Ebola hemorrhagic fever, and Zika

Natural killer (NK) cells are lymphocytes that are critical to innate and adaptive immunity[6]. NK cells mainly induce cytotoxicity and promote cytokine production[7]. Cytokines are immune signalling molecules that stimulate the maturation of dendritic cells, active monocytes, and cytotoxic T cells, or induce activation of B cells to enhance the immune response[8]. Activated NK cells produce various pro-inflammatory cytokines such as interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin (IL)-2 that contribute to the elimination of abnormal cells[9,10]. IFN-γ regulates major histocompatibility complex-1 expression to further activate CD8+ T cells to recognize tumor cells[11]. TNF-α acts as a tumor-promoting factor and enhances the production of IL-1β and IL-6 and stimulates nuclear factor-kappa B and signal transduction and activation of transcription inflammatory cascades[12]. IL-2 increases T cell proliferation and TGF-β production by NK cells[13,17]. Risky behaviors stimuli such as alcohol and tobacco abuse, antibiotics, chemotherapy, birth control pills, and other drug therapies can suppress immunity, particularly NK cell activity, which may further result in cancers or infections[14].

Herbs have long been used to prevent and treat many diseases. The beneficial effects of the different herbs are due to their active compounds composition and content[15]. Therefore, several formulations comprising a mixture of different herb types exert various pharmacological effects with less side effects[16]. Cornus officalis (C. officinalis) Sieb. et Zucc. (Cornaceae) is a deciduous tree native to eastern Asia and first recorded in Shen Nong’s Materia Medica (Shen-Nong-Ben-Cao-Jing) about 2000 years ago for the treatment of kidney diseases and diabetes[17]. Long-term use of C. officinalis may regulate the immunity and provide renal and neural protection. It contains abundant triterpenoids, iridoids, flavonoids, tannins, organic acids, and polysaccharides[18]. Loquat Eriobotrya japonica Lindl. (E. japonica) is a subtropical evergreen tree and is extensively used in traditional formulations[19]. E. japonica has antitussive, anti-inflammatory, and anticancer properties. According to previous studies, the leaf extract of Loquat increased serum IFN-γ and TNF-α levels in healthy mice[20,21]. Olive tree [(Olea europaea L., O. europaea)] is widely used in folk medicine in Europe[22]. Different forms of olive are appreciated globally by consumers who are health conscious. Its leaf extract mainly contains phenolics and two secoiridoid compounds: oleacein and oleuropein[23]. It is beneficial in the treatment of AIDS, chronic diseases, and melanomas by enhancing immunity[24,25]. Therefore, it is hypothesized that the herb formulae by combining these herb extract may exert more effective for treatment.

For more than 50 years, cyclophosphamide (CTX) has been widely used to treat various forms of cancers, including lymphoma, breast cancer, and leukemia[26]. However, CTX therapy is often restricted due to its toxicity and adverse effects. Therefore, CTX-induced immunosuppression mouse models have been used to determine the immunomodulatory effects of various herbs[27,28]. We evaluated the immune enhancement effect of an herb complex extract (HCE) comprising C. officinalis, E. japonica, and O. europaea in a cyclophosphamide-induced immunosuppression rat model.

2. Materials and methods

2.1. HCE preparation

The leaf extract of C. officinalis, E. japonica, and O. europaea was supplied by Teazen Co. (Anyang, Gyeonggi-do, Korea). Briefly, the leaves of herbs were dried at 60 °C and powdered in a blender. The dried powders were then extracted twice using 70% ethanol at 70 °C for 3 h. After being filtered, each extract was evaporated in a rotary evaporator and freeze-dried. For the mixture of HCE extracts, each extract was mixed together in a 1:1:1 ratio.

2.2. Cell culture

The spleens of Sprague-Dawley rats were harvested, and a single cell suspension of splenocytes was prepared using tweezers and a mesh. The suspension was washed three times by centrifugation (1000 × g, 5 min, 4 °C) with RPMI-1640 medium. The suspension was treated with a red blood cell lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) for 3 min to remove red blood cells. YAC-1 T cell lymphoma cells obtained from the Korea Cell Line Bank (KCLB40071) were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C and 5% CO2.

2.3. Cell proliferation assay

WST-1 assay kit (ITS Bio, Seoul, Korea) was used to investigate the effects of HCE (1–1000 μg/mL) on cell proliferation. Splenocytes (1 × 106 cells/90 μL/well) and YAC-1 cells (1 × 105 cells/90 μL/well) were cultured for 24 h and treated with HCE at different concentrations. The cells were incubated at 37 °C and 5% CO2 for 24 h and 48 h, and cell proliferation was evaluated by measuring the absorbance at 405 nm with a Multi Detection Reader (Infinite 200; TECAN Group Ltd., Männedorf, Switzerland) after adding 10 μL of WST-1 solution (per 100 μL cell medium) and incubation for 1 h. Cell proliferation rate (%) was calculated as (Absorbance of the sample treatment group/Absorbance of the control group) × 100%.

2.4. NK cell activity assay

The splenocytes were seeded at a density of 5 × 105 cells/mL in a 96-well plate, after which HCE (0, 1, 5, 10, 30, 50, 100, 200 μg/mL) were added to the wells. YAC-1 cells (1 × 105 cells/mL) were added to the effector cells and target cells at a ratio of 1:50, and then cultured in a 5% CO2 incubator at 37 °C for 24 h. After incubation, lactate
dehydrogenase was measured using a CytoTox detection kit (Takara Bio, Shiga, Japan). Formazan formed by the oxidation of nicotinamide adenine dinucleotide (NAD) in the reaction solution was measured at 490 nm and compared with that in the control group.

2.5. Experimental animals

Specific pathogen-free, 5-week-old Wistar rats were purchased from Sam Taco Bio Korea (Osan, Korea). During the acclimation period, the rats were allowed unrestricted access to a general solid diet (Samtako, Gyunggi, Korea) and filtered water. The housing environment was maintained at (23 ± 1) °C, (50 ± 5)% relative humidity, a noise level < 60 dB, and a 12-h photoperiod (08:00 to 20:00) with the illumination at an intensity of 150–300 Lux. This experiment was carried out in accordance with the experimental animal guidelines of Wonkwang University (Approval number: AKU17-66).

2.6. CTX–induced immunosuppression

CTX was purchased from Sigma-Aldrich and used at 5 mg/kg in the present study. CTX and HCE were coadministered. CTX was orally administered (5 mg/kg) after dissolving in distilled water.

2.7. Experimental design

During the acclimation period, experimental animals were separated into groups based on body weight using the randomized block design method as described previously with some modifications[29,30]. The experimental design involved normal, control (CTX 5 mg/kg body weight), red ginseng extract 800 mg/kg body weight (RGE) groups as reference group. The HCE groups were administered HCE at 200 mg/kg body weight (HCE 200), 400 mg/kg body weight (HCE 400) or 800 mg/kg body weight (HCE 800) which was selected from preliminary experiment for mice survival. Mice were divided into six groups with 10 rats per group. Individual rats were identified using ear punches.

2.8. Biomarker analysis

Body weights were measured weekly. Food and water intake were also measured weekly by measuring the amount of food and water remaining on the next day. After being anesthetized with diethyl ether, blood was collected from the abdominal vein for analysis, and liver, thymus, and spleen tissues were isolated and weighed. Spleen tissue was fixed in formalin for histological analysis.

2.9. Complete blood cell (CBC) count

Blood drawn from the abdominal vein was collected in EDTA-coated tubes (DB Caribe, Ltd., USA) and in conical tubes. Hematologic testing was performed using a blood analyzer (Hemavet 950 Fs; Drew Scientific, Dallas, TX, USA) after rotating the EDTA tubes containing the blood samples in a roll mixer for about 30 min.

White blood cells, lymphocytes, and neutrophils were quantified. Blood collected in the conical tube was used for cytokine analysis. After coagulation at room temperature for 30 min, serum was collected by centrifugation at 1 200 × g for 10 min. Separated serum was analyzed by an ELISA kit (Biolegend, San Diego, CA, USA) to measure TNF-α, IFN-γ, and IL-2.

2.10. Histological analysis

The isolated spleen tissue was fixed in 10% formalin solution, embedded in paraffin, and sliced into 4-μm-thick sections. The sections were treated with xylene to remove the paraffin, dehydrated using a graded ethanol series, and then stained with hematoxylin for 4 min and eosin for 2 min. The stained tissue sections were observed and photographed using a BX50 F4 optical microscope (Olympus, Tokyo, Japan).

2.11. Statistical analysis

The data are represented as Mean ± SD of triplicate measurements. Statistical analysis was performed by ANOVA followed by Dunnett’s post hoc test using the SPSS statistical program (version 12.0, SPSS Inc., Chicago, IL, USA). Significance was at $P<0.05$.

3. Results

3.1. Cell proliferation

Splenocytes and Y AC-1 were treated with 1–1 000 μg/mL HCE. Cell viability was measured at for 24 h and 48 h. Splenocyte viability decreased at doses ≥ 200 μg/mL at 24 and 48 h (Table 1). Y AC-1 viability decreased at doses ≥ 500 μg/mL at 24 and 48 h (Table 1). The maximum HCE concentration for NK cells used in subsequent experiments was 200 μg/mL.

Table 1

<table>
<thead>
<tr>
<th>Concentration of HCE (μg/mL)</th>
<th>Splenocytes 24 h</th>
<th>YAC-1 24 h</th>
<th>Splenocytes 48 h</th>
<th>YAC-1 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.1±0.1</td>
<td>100.1±0.1</td>
<td>100.1±0.1</td>
<td>100.1±0.1</td>
</tr>
<tr>
<td>1</td>
<td>110.2±2.4</td>
<td>102.1±1.6</td>
<td>99.9±0.3</td>
<td>99.2±0.3</td>
</tr>
<tr>
<td>5</td>
<td>115.1±1.4</td>
<td>103.1±1.9</td>
<td>99.2±0.2</td>
<td>101.0±0.3</td>
</tr>
<tr>
<td>10</td>
<td>116.1±3.2</td>
<td>106.2±1.5</td>
<td>100.5±0.2</td>
<td>99.2±0.3</td>
</tr>
<tr>
<td>25</td>
<td>112.6±3.4</td>
<td>106.1±3.4</td>
<td>100.1±0.4</td>
<td>100.1±0.3</td>
</tr>
<tr>
<td>50</td>
<td>112.1±3.2</td>
<td>104.2±3.0</td>
<td>101.5±0.6</td>
<td>101.0±0.1</td>
</tr>
<tr>
<td>100</td>
<td>113.2±3.2</td>
<td>102.3±1.5</td>
<td>101.9±0.6</td>
<td>101.0±0.2</td>
</tr>
<tr>
<td>200</td>
<td>99.6±3.0</td>
<td>89.3±2.4</td>
<td>100.1±0.6</td>
<td>103.6±0.2</td>
</tr>
<tr>
<td>300</td>
<td>94.4±3.6</td>
<td>62.8±3.5</td>
<td>97.8±0.4</td>
<td>103.8±0.8</td>
</tr>
<tr>
<td>400</td>
<td>82.4±0.8</td>
<td>49.8±2.7</td>
<td>97.8±0.4</td>
<td>102.4±1.6</td>
</tr>
<tr>
<td>500</td>
<td>73.3±3.1</td>
<td>51.4±1.4</td>
<td>101.7±1.2</td>
<td>68.6±2.6</td>
</tr>
<tr>
<td>1 000</td>
<td>19.7±0.1</td>
<td>6.1±4.8</td>
<td>12.5±1.0</td>
<td>5.6±0.1</td>
</tr>
</tbody>
</table>

The results are expressed as mean±SD. $P<0.05$ comparing with control group.
3.2. NK cell activity

The activity of NK cell treated with HCE began to increase at 10 μg/mL in HCE treatments. At the maximum concentration of 200 μg/mL, the activity increased by 48.5% compared to control.

3.3. Variation in body weight and food and water intake

After oral administration experiment, no mice died after dosing. Compared with the normal group treated with distilled water only, the control group treated with CTX alone displayed decreased body weight gain. At the end of the experiment (4 weeks), the weights were (240.2 ± 5.3) g in the normal group and (238.8 ± 3.0) g in the CTX group. In the HCE 200, 400, and 800 groups, they were (237.2 ± 3.6) g, (236.3 ± 3.8) g, and (233.0 ± 5.7) g, respectively. Body weight in the RGE 800 group was (246.9 ± 3.6) g. No significant difference was found between the experimental groups. HCE treatment did not significantly affect food and water intake in the CTX-immunosuppressed rats.

3.4. Liver, spleen, and thymus weights

Liver weight was significantly lower in the control group (6.75 ± 0.04) g than in the normal group (8.01 ± 0.09) g. No significant difference in liver weight was observed between the HCE 200 and HCE 400 groups. In the HCE 800 group, the liver weight was (7.25 ± 0.06) g, which was not significant different from the RGE 800 group (7.40 ± 0.05) g (Table 2).

Spleen weight was (0.55 ± 0.01) g and (0.37 ± 0.01) g in the normal and control groups, respectively, indicating that CTX decreased spleen weight. The spleen weights in the HCE treatments differed slightly from that in the CTX group (Table 2).

The thymus tissue weight was (0.31 ± 0.01) g in the CTX group and (0.54 ± 0.01) g in the normal group. The weights in the HCE 400 group [(0.37 ± 0.01) g] and HCE 800 group [(0.36 ± 0.01) g] were similar to that in the RGE 800 group (0.36 ± 0.01) g; these weights were significantly higher than that in the CTX group (Table 2).

Table 2

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Liver weight</th>
<th>Spleen weight</th>
<th>Thymus weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>8.01 ± 0.09</td>
<td>0.55 ± 0.01</td>
<td>0.54 ± 0.01</td>
</tr>
<tr>
<td>Control</td>
<td>6.75 ± 0.04</td>
<td>0.37 ± 0.01</td>
<td>0.31 ± 0.01</td>
</tr>
<tr>
<td>200 mg/kg HCE</td>
<td>7.13 ± 0.02</td>
<td>0.38 ± 0.01</td>
<td>0.35 ± 0.03</td>
</tr>
<tr>
<td>400 mg/kg HCE</td>
<td>7.21 ± 0.02</td>
<td>0.41 ± 0.03</td>
<td>0.37 ± 0.01</td>
</tr>
<tr>
<td>800 mg/kg HCE</td>
<td>7.25 ± 0.06</td>
<td>0.45 ± 0.03</td>
<td>0.36 ± 0.01</td>
</tr>
<tr>
<td>800 mg/kg RGE</td>
<td>7.40 ± 0.05</td>
<td>0.42 ± 0.01</td>
<td>0.36 ± 0.01</td>
</tr>
</tbody>
</table>

The results are expressed as mean ± standard deviation. *P<0.05 comparing with normal group; †P<0.05 comparing with control group; Abbreviations: HCE=herb complex extract; RGE=red ginseng extract.

3.5. CBC analysis

White blood cell count was significantly lower in the CTX group than in the normal group. However, no significant difference was found between the experimental groups except for HCE 200 group (Table 3). Lymphocyte count was significantly lower in the control group and HCE groups than in the normal group. No significant difference was found between the experimental groups (Table 3). Neutrophil count in the normal group was higher than that in the CTX group, indicating that CTX significantly reduced neutrophil count. No significant difference was found between the experimental groups (Table 3).

Table 3

<table>
<thead>
<tr>
<th>Treatments</th>
<th>White blood cell</th>
<th>Lymphocyte</th>
<th>Neutrophil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>6.91 ± 0.01</td>
<td>4.71 ± 0.02</td>
<td>1.88 ± 0.02</td>
</tr>
<tr>
<td>Control</td>
<td>2.67 ± 0.01</td>
<td>1.49 ± 0.02</td>
<td>0.69 ± 0.02</td>
</tr>
<tr>
<td>200 mg/kg HCE</td>
<td>2.77 ± 0.02</td>
<td>1.92 ± 0.02</td>
<td>0.82 ± 0.01</td>
</tr>
<tr>
<td>400 mg/kg HCE</td>
<td>2.93 ± 0.02</td>
<td>1.93 ± 0.02</td>
<td>0.98 ± 0.01</td>
</tr>
<tr>
<td>800 mg/kg HCE</td>
<td>2.97 ± 0.02</td>
<td>1.86 ± 0.02</td>
<td>0.91 ± 0.02</td>
</tr>
<tr>
<td>800 mg/kg RGE</td>
<td>3.26 ± 0.02</td>
<td>2.19 ± 0.02</td>
<td>0.92 ± 0.03</td>
</tr>
</tbody>
</table>

The results are expressed as mean ± SD. *P<0.05 comparing with normal group; †P<0.05 comparing with control group; Abbreviations: HCE=herb complex extract; RGE=red ginseng extract.

3.6. Serum cytokine analysis

Serum TNF-α content in the normal group was significantly higher than that in the CTX group. In the HCE 200 and HCE 400 groups, there were not significantly different from that in the CTX group. However, it was significantly higher in the HCE 800 group (9.45 ± 0.63) pg/mL and the RGE 800 group (10.28 ± 0.26) pg/mL than in the CTX group (Table 4). The same pattern appears in IL-2 cytokine levels. No significant change in IFN-γ production was observed in HCE treated groups as compared with control group.

3.7. Spleen histological analysis

Optical microscopy was used to investigate the effect of HCE on spleen histology. In the normal group, white pulp was uniformly distributed around the central vein. In addition, the lymph node was surrounded by the marginal zone, and the boundary with the red pulp was clearly distinguished (Figure 1A). However, with CTX treatment, the boundary of the edge zone was unclear, and a remarkable decline in white pulp was observed, and the red pulp was condensed with an irregular arrangement (Figure 1B). HCE 200 did not significantly improve spleen histology. The marginal zone was localized (Figure 1C). In the HCE 400 group, the edge zone around the red pulp was clearly distinguished, unlike that in the control group, where breakdown of white pulp was not observed (Figure 1D). In particular, in the HCE 800 group, breakdown of white pulp by CTX treatment and the condensation of red pulp greatly improved. Overall, the marginal zone of the tissue was clearly observed (Figure 1E). In the RGE 800 group, compared to that in the control group, breakdown of white pulp was not observed, and the condensation of red pulp was greatly alleviated (Figure 1F), similar to that in the HCE 800 group.
and anti-inflammatory properties[31,32]. and olive leaves. RGE, which have found application in antioxidant

This study was conducted to investigate the immunomodulatory effect of HCE comprising

The results are expressed as mean ± standard deviation. *P<0.05 comparing with normal group, †P<0.05 comparing with control group; Abbreviations: HCE=herb complex extract; RGE=red ginseng extract.

Figure 1. Spleen tissue H & E staining.

(A) normal, (B) cyclophosphamide 5 mg/kg, (C) herb complex extract 200 mg/kg (HCE 200), (D) herb complex extract 400 mg/kg (HCE 400), (E) herb complex extract 800 mg/kg (HCE 800), and (F) red ginseng extract 800 mg/kg (RGE 800). Red ginseng is the positive control.

4. Discussion

This study was conducted to investigate the immunomodulatory effect of HCE comprising C. officinalis S. et Z., E. japonica Lindley, and olive leaves. RGE, which have found application in antioxidant and anti-inflammatory properties[31,32].

First, we studied the effect of HCE on NK cell activity. NK cell activity was measured in splenocytes and YAC-1 cells. HCE increased the activity of NK cells by about 38% at the maximum treatment concentration of 100 μg/mL. In many previous studies, the increasing of NK cell activity may enhances natural immunity[10,33]. The NK cell could be responsible for changing organs and activating of various cytokines[28,34].

With oral administration treatment, we investigated the immunity-improving effect of HCE in immunosuppressed rats. Administration of CTX reduced the weights of the liver, spleen, and thymus, indicating its direct toxicity[35]. However, HCE attenuated this decrease. The same results also noticed in previous studies that C. militaris extract increased spleen weight[36]. These findings constitute indirect evidence that HCE modulated body immune responses.

Generally, the mice enhanced immunity have been reported to increase CBC levels[34]. Cyclophosphamide is an immunosuppressant cause suppression of CBC[37]. In this study, the proliferation of leukocytes, lymphocytes, and neutrophils was enhanced significantly higher than those of in the CTX-treat group. Some results have been found in some herbal medicines[28,37]. Moreover, although HCE 800 showed negative effects on splenocytes and YAC-1, the oral administration of rat with HCE 800 treatment indicated that there was no influence on mouse survival rate.

Cytokines are involved in immune regulation. In this study, administration of CTX decreased cytokine levels. However, TNF-α and IFN-γ levels were recovered by HCE treatment. IL-2 also increased. Especially, HCE at 800 mg/kg showed similar effects to RGE at 800 mg/kg. TNF-α is involved in the regulation of macrophages and T cells[38]. IFN-γ modulates IL-2 activity, which affects NK and T cells[39]. CTX is known to cause immunosuppression by damaging DNA of T and B cells[40]. In this study, the administration of HCE attenuated the decrease in cytokine levels. Therefore, the suppressed immune response improved through the activation of NK and T cells.

Improvement in spleen histology is associated with immunity enhancement[41]. The atrophic changes in CTX group was confirmed by histomorphometrical analysis in this study. We found that spleen histology in the HCE 800 group was similar to that in the normal group. These results are direct evidence that HCE exhibits potent immunomodulatory effects through activated NK cells and promoting cytokine on CTX-induced mice.

In conclusion, the in vitro and in vivo results show that HCE enhances immunity by increasing NK cell activity, regulating cytokine levels, and maintaining spleen weight. Therefore, it may be used as a potential immunity enhancer.

Conflict of interest statement

We declare that we have no conflict of interest.

Foundation project

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