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DEU-7 Derived from *Ulmus macrocarpa* Improved Immune Functions in Cyclophosphamide-treated Mice

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The present study investigated the immunomodulatory properties of four different medicinal plants in a cyclophosphamide-treated Balb/c mouse model. One of the four plants, *Ulmus macrocarpa*, showed partial resistance against immune suppression induced by cyclophosphamide. The bark of *U. macrocarpa,* commonly known as the Chinese elm, has been used as a pharmaceutical material in Korean traditional medicine to treat bacterial inflammation and induce wound healing. In this study, water extract of *U. macrocarpa,* named DEU-7, was used for its immunomodulating functional activity. DEU-7 increased the weight of the spleen and the number of splenocytes but did not significantly affect the liver, kidney, and thymus *in vivo*. A splenocyte viability assay confirmed that DEU-7 influenced *ex vivo* splenocyte survival. DEU-7 also increased the levels of cytokines, such as IL-2 and IL-4, and immunoglobulins, such as IgM, IgG, and IgA. These results indicated that DEU-7 is involved in the activation of T and B lymphocytes. In addition, DEU-7 was able to maintain the production of cytokines, such as TNF-α, IL-12, and IFN-γ, in the condition of cyclophosphamide-induced immune suppression, suggesting that DEU-7 activated innate immune cells, even under immune suppression. We concluded that DEU-7 aids immunological homeostasis, thereby preventing immune suppression, and aids both innate and adaptive immune response by maintaining the levels of various cytokines and immunoglobulins. Consequently, it is worth investigating the potential of DEU-7 as a supplemental source for immune-enhancing agents.

Key words : Cytokines, immunomodulator, immunoglobulins, immunosuppression, *Ulmus macrocarpa*

Introduction

The immune response is categorized as innate immunity and acquired immunity. Innate immunity is a non-specific response that is characterized by phagocytosis and is also called natural immunity [31]. Acquired immunity is a specific immune response by B and T cells. In contrast to innate immunity, it can effectively inhibit the repetitive invasion of the same antigen, which is due to the memory function generated by the intrusive antigen. Innate immunity and acquired immunity are closely related to coordinated host defense mechanism against pathogens [20, 23, 31]. This study aims to find a new substance that can be a potential therapeutic agent against aging diseases by enhancing the immune response, which must be increased as a human being grows older. We selected four different kinds of medicinal plants known to enhance immune function in traditional folk medicine. *Alpinia officinarum* is a medicinal plant that assists the digestive function that is called galangal root. It has been known to inhibit the growth of hemolytic streptococcus and diphtheria [11, 28]. *Zanthoxylum schinifolium,* Chinese pepper, is used mainly as a spice and has been known to sterilize and detoxify. But recent studies revealed that it has anti-cancer effects [30]. *Scutellaria baicalensis* Georgi has an anti-inflammatory effect [19], and many stud-

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ies showed the results of the therapeutic effects on lung, ovarian and uterine cancer [6, 21]. *Ulmus macrocarpa,* known as the Chinese elm, has been used in traditional folk medicine to treat skin wounds and anti-inflammation [17, 18]. Water extracts of these four medicinal plants were designated to DEU-2, 3, 4 and 7 and we investigated whether DEU-2, 3, 4 and 7 have an effect on immune responses in an immune suppressed mouse model.

Cyclophosphamide is efficient in inhibiting the immune system and is used in the treatment of cancer. It has been known to reduce the production of lymphocytes in the spleen and lymph node. The appropriate treatment concentration of cyclophosphamide was determined based on the various experimental studies [4, 7, 12, 16]. An i*n vivo* test was performed by feeding the mice DEU-2, 3, 4 and 7 once a day. One of the four DEU extracts, DEU-7 affected changes in the weight of the spleen and production of cytokines and immunoglobulins. Many studies suggested previously that *Ulmus macrocarpa* has anti-inflammation effects [8, 15, 23] and anti-cancer effects [1, 14]. However, most studies have not suggested the mechanism influenced by *Ulmus macrocarpa*. Therefore, we investigated the mechanism by DEU-7 in immunomodulating function and propose the possibility of its use in immune enhancing substances.

Materials and Methods

Animals

Male Balb/c mice were purchased from Samtako, Inc. (Osan, Korea). Mice used in all experiments were 12 weeks old. These mice were housed in a specific pathogen-free facility with the appropriate temperature and humidity, and allowed free access to food and water. The mice for this study (DEU-R2013-002) were approved by the Institutional Animal Care and Use Committee at Dong-Eui University.

Preparation of DEU extracts

Dried samples of *Alpinia officinarum*, *Zanthoxylum schinifolium*, *Scutellaria baicalensis* Georgi, *Ulmus macrocarpa* Hance were purchased from Dae-Han herbal medicine Inc. (Busan, Korea), and the samples were stored in Dong-Eui University (No, 20140204). Three kilograms of dried samples were added to 10 L of water, and extracted twice repeatedly for 3 hr at 80℃. The extract was filtered and concentrated under reduced pressure at below 45℃ using the Rotary vacuum evaporator (Eyela, Japan). The concentrates were dried using a freeze-dryer at -80℃, resulting in about 320 g of water extracts obtained. We labeled each *Alpinia officinarum*, *Zanthoxylum schinifolium*, *Scutellaria baicalensis* Georgi, and *Ulmus macrocarpa* extract as DEU-2, 3, 4 and 7, respectively.

In vivo Immunomodulation test of the immunosuppressive mouse model

Six mouse groups (n=6 in each group) were divided. One control (normal) group was without any treatment. Another group was treated with only cyclophosphamide (cyclophosphamide group) as an immunosuppressant. Four other groups were treated with cyclophosphamide and DEU-2, 3, 4 or 7 (DEU group). On day 0, five groups were intraperitoneal injected with cyclophosphamide at a dose of 100 mg/kg, and the normal group was injected with saline (10 ml/kg). Feeding of DEU groups started from day 1 with a 300 mg/kg dose and was carried out until day 14. The normal and cyclophosphamide groups were fed 0.5 ml distilled water per day. Body weights of mice were measured once in three days, and on day 15 organs were removed from all mice. Each organ was weighed, and splenocytes were separated from spleens using mesh screen and RBC lysis. The RBC lysis buffer was purchased from the Biolegend Inc. (CA, USA). Splenocytes were cultured in RPMI 1640 media and then used in the viability assay. Body weights of mice were presented as the body index by statistical analysis.

Cell viability assay

Mouse splenocytes were seeded into a 96 well plate at a density of 1×10^6 cells/ml. Cells were treated with phytohemagglutinin (PHA, 10 μg/ml), lipopolysaccharide (LPS, 20 μg/ml) or DEU-7 extract (200 μg/ml) for 48 hr at 37℃ CO_2 incubator. Live cells at each time point were measured by MTT assay. Briefly, the Cell Titer 96®AQ_{ueous} One Solution Reagent (Promega, USA) was added to each well of 96 well plates. The plate was incubated for 3 hr at 37℃, and then measured for absorbance at 490 nm.

Measurement of cytokine levels and immunoglobulins

The levels of total interleukin (IL)-2, IL-4, IL-6, IL-10, IL-12, interferon-gamma (IFN-γ), and TNF-α in blood plasma of mice were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences Pharmingen, CA, USA) following the manufacturer's instructions. The concentrations of each cytokine were calcu-

lated from the co-responding standard curves. Immunoglobulin levels were measured similarly using commercially available ELISA kits (Immunology Consultants Laboratory Inc., OR, USA).

Statistical analysis

Statistical analysis was determined by three independent experiments and each experiment had eight mice per group. *P*-values were determined through the one-way ANOVA, and less than 0.01 were considered statistically significant. *P*-values represented as an asterisk (*) or #.

Results

DEU-7 increased viability of mouse splenocytes

To examine the function of DEU extracts derived from four kinds of plants, *in vivo* test was performed for 15 days. On day 0, all mouse groups except for the normal group were injected with cyclophosphamide to suppress the immune response. Mice were then fed the DEU extracts at a dose of 300 mg/kg for two weeks. Body weights of mice were measured once every three days, but there was no significant difference in the experimental groups (Fig. 1). At 15 days, mice were dissected and their organs weighed. The weights of liver and kidney were unchanged, but the spleen weight was changed by DEU-3 and DEU-7 (Fig. 2). To confirm the results of the spleen, the splenocytes were separated from spleen. The results of the splenocyte counting were higher in the DEU-7 group but not the DEU-2, 3, 4 groups (Fig. 3A). In order to confirm these results, the splenocytes were separated from normal mice and treated with PHA and LPS as a positive control and DEU-7. Viability assay was then performed. As a result, viability of splenocytes was greater by DEU-7 over 48 hr when compared to the normal group (Fig. 3B). These results suggested that the change of spleen index by DEU-7 treatment is closely associated with the increase of splenocyte number.

DEU-7 increased the levels of cytokines and immunoglobulins

Spleen is a secondary lymphoid organ and composed of many immune cells such as T, B, dendritic cells and macrophages. We tested activation of these cells by measuring cytokines and immunoglobulins. Cytokine and immunoglobulin levels in the blood were measured by ELISA. DEU-7 noticeably increased the levels of IL-2, IL-4, IL-6 and IL-12 in the blood and an increase of IFN-γ was shown. Other DEU extract groups showed similar cytokine levels with the cyclophosphamide group (Fig. 4). The results of DEU-7 suggested that it has a sufficient role to maintain T

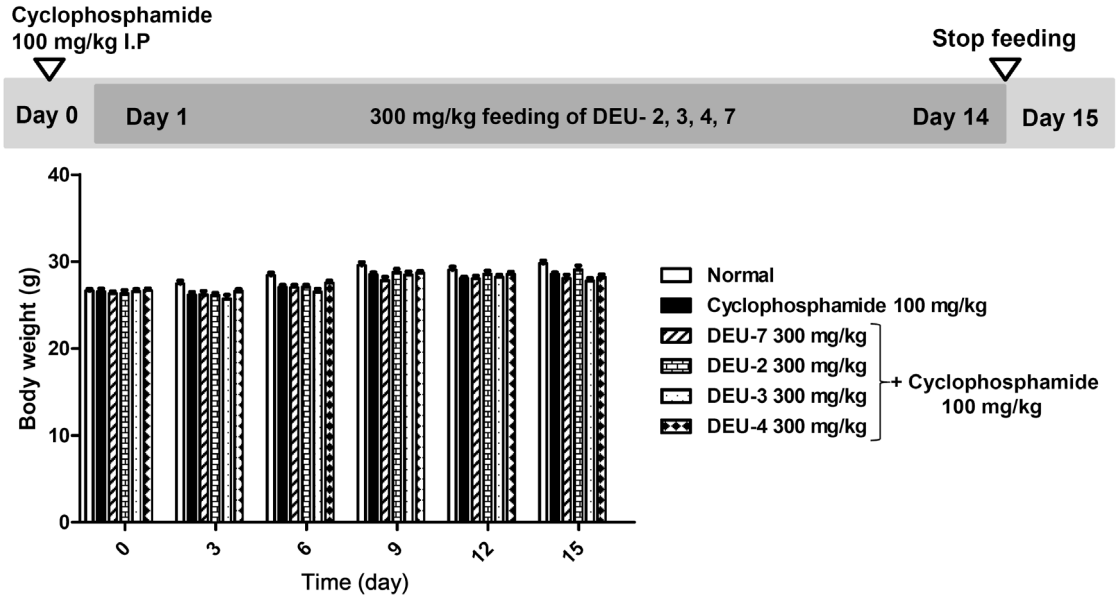

Fig. 1. A comprehensive overview of *in vivo* test design for 15 days. Six mouse groups were divided. One normal group was without any treatment. Another group was treated with only cyclophosphamide. Four other groups were treated with cyclophosphamide and DEU-2, 3, 4 or 7. On day 0, five groups except the normal group were injected i.p. with 100 mg/kg of cyclophosphamide. Each DEU-2, 3, 4, and 7 group was fed at a dose of 300 mg/kg/day for 14 days. Body weight was measured once every three days. Six mice per group used.

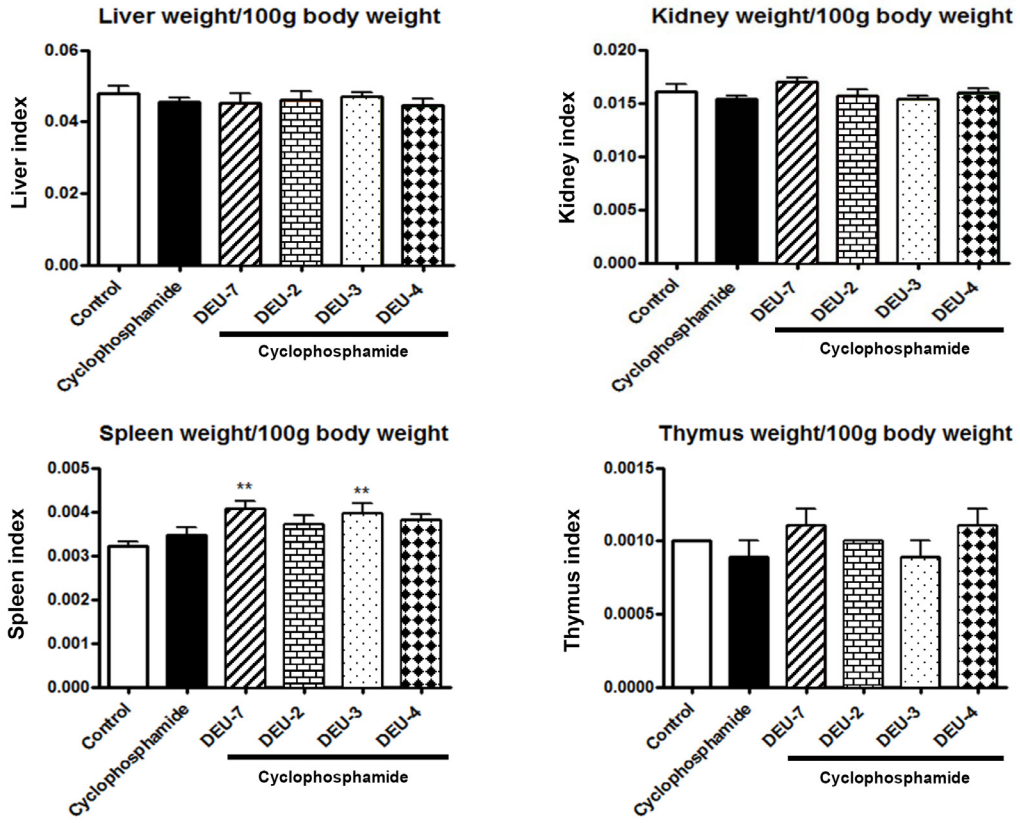

Fig. 2. DEU-7 influenced the organ index. At 15 days, mice were dissected. The organ weights were measured and calculated for the organ index. DEU-7 increased the weights of the spleen. *P* values are indicated by $^{**}p<0.001$ compared to the control.

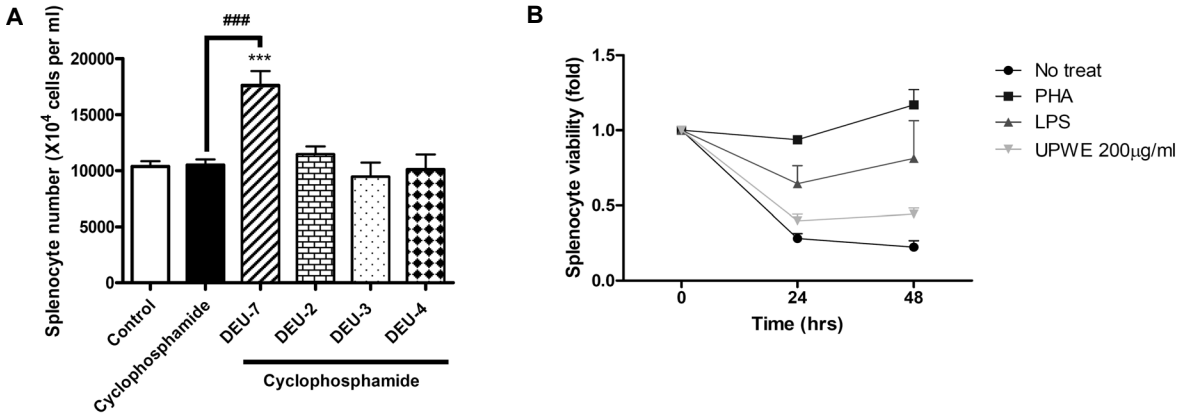

Fig. 3. DEU-7 increased splenocyte proliferation. (A) After dissection, the spleen was removed and splenocytes were counted using a hemocytometer. (B) DEU-7 increased *in vitro* viability of splenocytes. All experiments were performed in triplicate and repeated twice. *P* values are indicated by *** $p<0.0001$ compared to the control. *P* values for cyclophosphamide represents ### $p<0.0001$.

cell mediated immunity because IL-2 and IL-4 affect on T cells. DEU-7 increased the levels of the immunoglobulins (Fig. 5). The levels of IgG and IgM were increased to the control level by DEU-7 treatment, and completely restored the level reduced by cyclophosphamide. IgA level was not statistically significant; however, mean value of DEU-7 was greater than IgA level of cyclophosphamide group. Therefore, DEU-7 has the effect of promoting B cell immunity under the condition of cyclophosphamide treatment.

DEU-7 recovered the reduction of immune response caused by cyclophosphamide

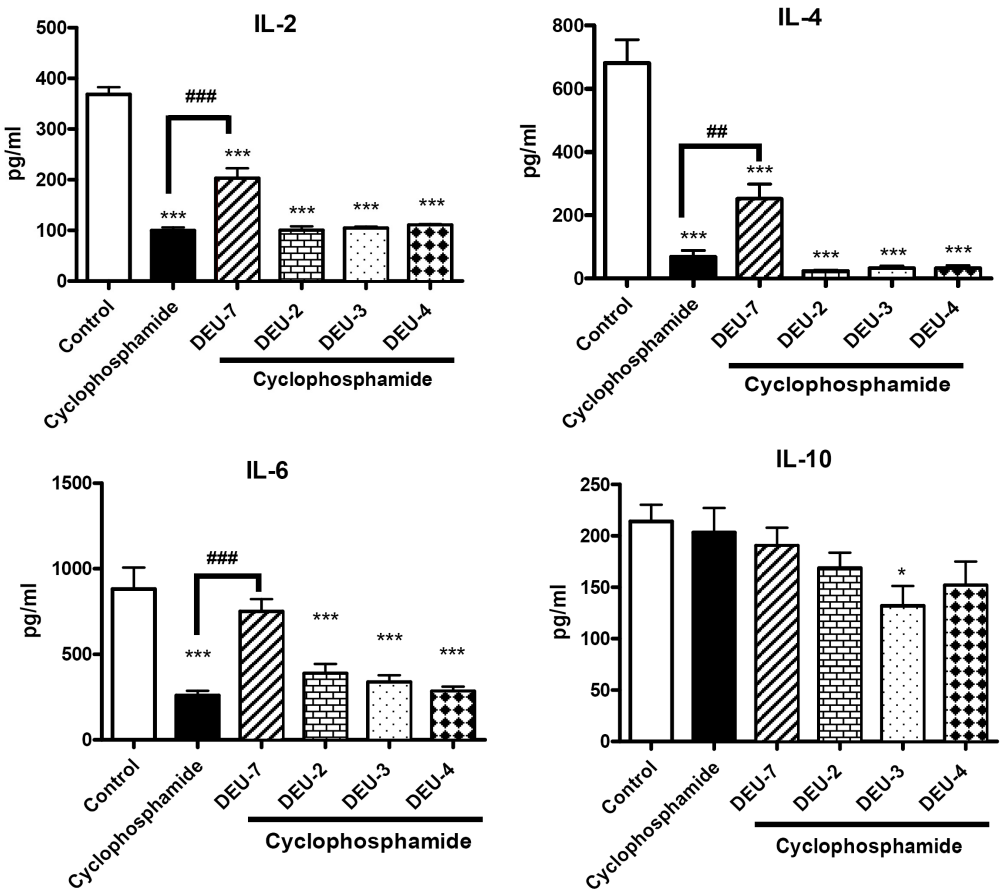

Fig. 4. DEU-7 increased cytokine levels. Cytokine levels in blood were measured using ELISA. DEU-7 increased the levels of cytokines related to B and T cell immunity under immunosuppressed condition. The levels of IL-2, 4, and 6 were significantly increased by DEU-7. *P* values are indicated by * $p<0.01$, *** $p<0.0001$ compared to the control. *P* values for cyclophosphamide represents ## $p<0.001$, ### $p<0.0001$.

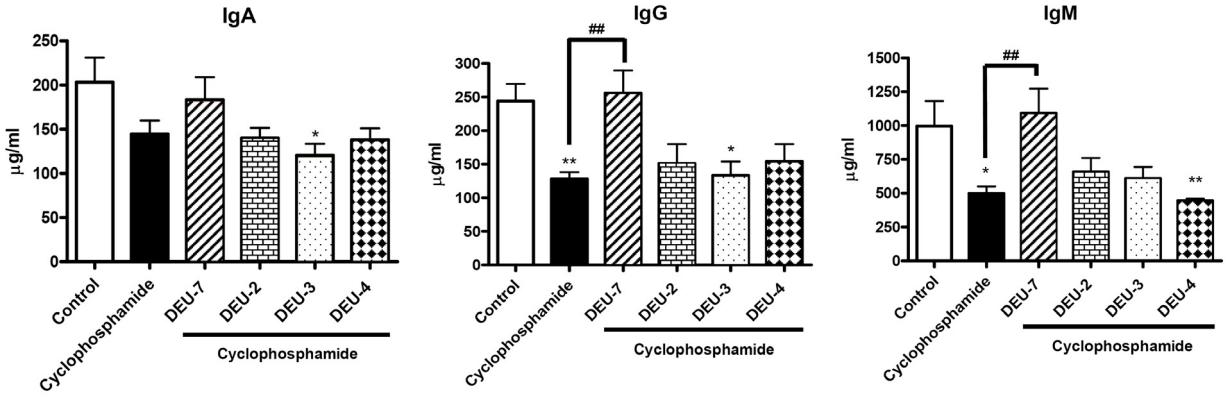

Fig. 5. DEU-7 increased the immunoglobulin levels in blood. Immunoglobulin levels were assayed using ELISA. DEU-7 recovered each IgA, IgG and IgM level to the normal levels in the immunosuppressed group. *P* values are indicated by *$p<0.01$, **$p<0.001$, compared to the control. *P* values for cyclophosphamide represents ##$p<0.001$.

Natural killer cell (NK cell) is one of major innate immune cells and its activation is regulated by TNF-α and IL-12. IFN-γ is the principal cytokine secreted by activated NK cells and its major function is to activate macrophages. Macrophages secret TNF-α and IL-12 and NK cells secret IFN-γ, which create a system of positive feedback in innate immune network. Cyclophosphamide reduced the levels of TNF-α, IL-12 and IFN-γ. However, DEU-7 restored the levels of

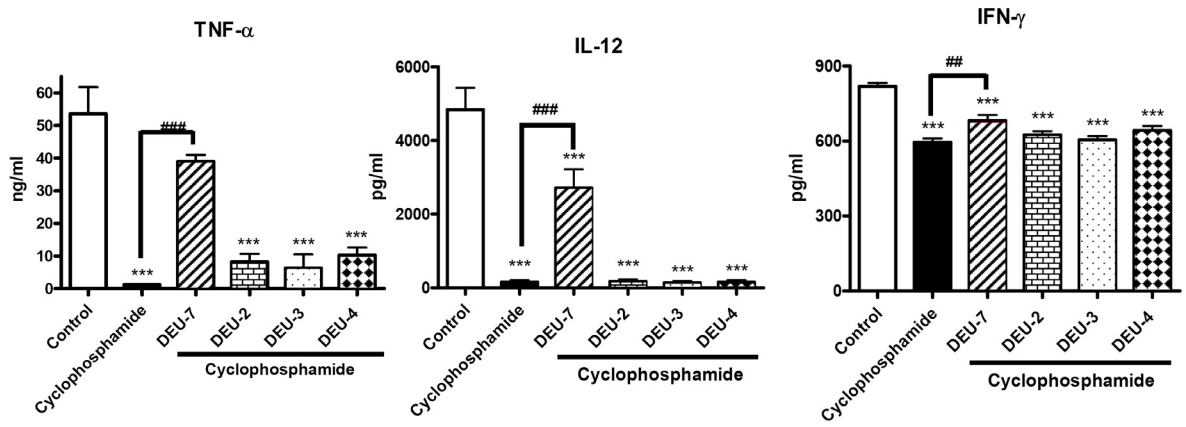

Fig. 6. DEU-7 increased NK cell-related cytokine levels that were reduced by cyclophosphamide. *P* values are indicated by ***p<0.0001 compared to the control. *P* values for cyclophosphamide represent ###p<0.0001.

TNF-α, IL-12 and IFN-γ unlike the other DEU extracts (Fig. 6). These data suggested that DEU-7 could have the ability to restore on the innate immune response inhibited by cyclophosphamide.

DEU-7, no other DEUs, did recover the levels of various cytokines and immunoglobulins to their normal levels closely through the stimulation of immune cells. Therefore, DEU-7 has the ability to maintain the homeostasis in the combination of innate immunity and adaptive immunity.

Discussion

Well-known traditional medicinal plants are good sources for screening functional compounds. We selected four different medicinal plants after literature survey and *in vitro* screening of a plant extracts library which was in-house prepared.

For this experiment, we used the immune-suppressed mouse model by cyclophosphamide treatment. Cyclophosphamide is a known immunosuppressant as well as an anti-cancer agent in low doses. The immune suppression by cyclophosphamide targets lymphocyte and neutrophil populations in the blood and the inhibition of macrophage migration [12, 26, 32]. The cyclophosphamide tends to decrease body weight of the experimental mouse group depending on its doses and injection times. Our experimental model was designed to mimic mild suppression of immune responses under normal condition. A single dose of 100 mg/kg cyclophosphamide IP administration did not cause body weight loss or organ indices but caused the levels of cytokines and immunoglobulins. Only DEU-7 appeared to prevent cyclophosphamide immune suppression. Interestingly, even under the cyclophosphamide treated condition DEU-7 increased the number of splenocytes suggesting not only protecting from cyclophosphamide but also stimulating immune cells in spleen. Also, DEU-7 partially recovered the levels of IL-2, IL-4, IL-6 and IL-12 which were reduced by cyclophosphamide. These cytokines are related to T cell mediated immunity, and involved in the T cell proliferation and differentiation [13, 20]. In addition, cytokines play roles in the B cell differentiation to the antibody secreting plasma cell [24, 29]. In consist with these results DEU-7 increased the levels of immunoglobulins equal to the normal levels under the cyclophosphamide treated condition. It implies that DEU-7 could be related to B cell differentiation and the antibody class switching [2, 33]. The spleen is the main place for B cell activation [3, 22, 27]. However, DEU-7 had no effect on IL-10 (Fig. 4). IL-10 has been found to act as the inhibitory factor of pro-inflammatory cytokines such as IL-2, TNF-α and IFN-γ [9]. Therefore, this result can support the increase of IL-2, TNF-α and IFN-γ by DEU-7.

NK cells are the killer lymphocytes of the innate immune response and comprise 5-25% of the lymphocytes in the blood. They are stimulated by IL-12 and TNF-α, both of which are produced by macrophages and release IFN-ɣ upon stimulation [10]. In this experimental model cyclophosphamide reduced the levels of these cytokines, which has been reported before [4, 13, 19]. DEU-7 restored the levels of IL-12 and TNF-α and IFN-ɣ under the cyclophosphamide treated condition. The recovery by DEU-7 treatment was not as much as the normal levels but significantly near to or half of the normal levels. These data suggested that DEU-7 would stimulate macrophages and in turn macrophages stimulate NK cells resulting keep the in-

nate immune working.

We had not solved yet how DEU-7 can maintain high levels of various cytokines or the normal levels of immunoglobulins because of early phase of *in vivo* study. *In vitro* mechanism study is undergoing at the present time. But it is clear that DEU-7 was able to maintain homeostasis of immune response under the immune-suppression drug treatment, which is promising the DEU-7 as a new therapeutic agent. In further study, we will establish the mechanism for promoting immune response by DEU-7.

Acknowledgement

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초록 : 면역억제 마우스 모델에서 왕느릅나무 유래 DEU-7의 면역기능 증강

강경화[1,5] · 고지수[1] · 이상호[1] · 이인환[2,5] · 이성도[2,5] · 김덕원[2,5] · 이종환[3,5] · 황혜진[4,5] · 현숙경[4,5] · 김병우[5] · 김철민[6] · 정경태[2,5]*

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고량강(*Alpinia officinarum*), 산초(*Zanthoxylum schinifolium*), 황금(*Scutellaria baicalensis* Georgi), 왕느릅나무(*Ulmus macrocarpa* Hance), 네 종류의 식물성 한방 약재의 면역증강효능을 cyclophosphamide를 처리한 동물모델을 이용하여 조사하였다. 실험은 cyclophosphamide로 면역억제를 유도한 후 네 종류의 한방 약재를 식이하여 cyclophosphamide에 의해 억제된 면역인자의 회복 여부를 조사하였다. 네 종류의 한방 약재를 동일한 방법으로 열수 추출한 후 동일한 농도로 마우스에 처리하였다. 이 중 왕느릅나무 열수 추출물(DEU-7)이 cyclophosphamide의 영향으로 면역 억제된 마우스에서 면역인자 혈중 농도를 정상치 준하는 또는 가깝게 유지하는 것으로 나타내었다. DEU-7에 의해 비장의 무게와 비장세포수는 증가하였으나 간과 흉선과 같은 다른 장기에는 통계적으로 유의한 변화가 없었다. *Ex vivo* 조건에서 DEU-7은 비장세포의 사멸을 지연시키는 것으로 나타났다. 중요한 면역인자인 IL-2와 IL-4 cytokine은 면역억제로 농도가 감소되었으나 DEU-7이 약 2배와 3배 향상시키는 것으로 나타났으며, 이는 정상치의 약 1/2 수준이었다. IgM과 IgG의 농도는 cyclophosphamide 처리로 정상치의 약 1/2로 떨어졌으나, DEU-7에 의해서 정상치와 동일한 농도로 증가하였다. IL-2와 IL-4 결과에서 DEU-7은 T 림프구에 영향을 줄 수 있다고 생각되며, 또한 IgM과 IgG의 결과로서 B 림프구에 영향을 줄 수 있다고 생각된다. 선천성 면역에 중요한 면역인자인 TNF-α, IL-12과 IFN-γ 역시 cyclophosphamide에 의해 농도가 감소되었으나 DEU-7에 의해 정상치에 가깝게 회복되었다. 따라서, DEU-7은 면역 억제 또는 감소된 상태를 정상 상태로 회복 또는 유지하는 기능이 있는 것으로 생각된다.