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Cyclophosphamide 유도 면역억제 Sprague-Dawley 동물모델에서 구절초추출물의 면역증진 효과

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Immune-boosting Effects of *Chrysanthemum zawadskii* Herbich var. latilobum Kitamura Extract on Cyclophosphamide-induced Immunosuppressed Sprague-Dawley Animal Model

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ABSTRACT

Background: *Chrysanthemum zawadskii* Herbich var. *latilobum* Kitamura extracts (CZE) is used to treat of various inflammatory and chronic diseases. Although commonly adminstered, no reports suggest its potential usage as an immune-booster.
Methods and Results: In the cyclophosphamide (CP, 5 ㎎/㎏)-induced immunosuppression model, the immune-boosting effect of CZE (oral administration for 2 weeks with CP) was evaluated by investigating cell proliferation, activity of natural killer (NK) cells, cytotoxic T lymphocytes, and production of CP-repressed cytokines [such as tumor necrosis factor-α, interferon-γ, interluekin (IL)-2, and IL-12] in isolated splenocytes. *In Vitro*, CZE treatment enhanced cell proliferation, activity of NK cells and cytotoxic T lymphocytes activity, and production of CP-repressed cytokines. *In Vivo*, CZE treatment promoted the production of white blood cells, lymphocytes, medium-sized cells, and granulocytes and proliferation of NK cells in CP-induced immunosuppressed mice. Additionally, the CZE treatment restored the TNF-α and IL-12 reduced by CP to normal levels and prevented spleen tissue damage.
Conclusions: CZE could be an effective immune-booster and potentially be added to functional foods to enhance immunity.

***Key Words*:** *Chrysanthemum zawadskii* Herbich var. *latilobum* Kitamura, Cyclophosphamide, Cytokine, Immune-boosting

INTRODUCTION

Chrysanthemum zawadskii Herbich var. *latilobum* Kitamura, known as “Gu-Jeol-Cho” in Korea, is a perennial herb Compositae family. It has been widely used as a traditional medicine and food for the treatment of gastroenteric disorders, bronchitis, pneumonia, pharyngitis, cough, bladder-related disorders, and hypertension (Han *et al.*, 2002).

C. zawadskii has been identified to various chemical components including quinon, linarin and acacetin (Chang and Kim, 2012; Shim *et al.*, 2012a; Shim *et al.*, 2012b). *C. zawadskii* has a various pharmacological property, including anti-cancer, anti-inflammatory, anti-oxidative, and liver-protective effects (Hsu *et al.*, 2004; Singh *et al.*, 2005; Shim *et al.*, 2012b; Seo *et al.*, 2010). However, it has not yet been studied about immune-boosting effects.

The immune system is a very complex and sophisticated network of immune molecules, immune cells, tissues, and

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organs to protect the body from external invasion of bacteria, parasites, viruses, and toxins (Spiering, 2015). The series of steps that act as a body defense against these infections are called immune reactions, and the development of cells involved in immune reactions occur in organs such as the thymus and spleen (Springer, 1990; Blackburn and Kellems, 2005).

Immune responses is highly complex biological responses involving numerous cytokines, growth factors and various immune cells such as white blood cell, lymphocyte, and granulocyte (Wynn *et al.*, 2013).

Lymphocytes are white blood cells comprising of T cells, B cells, and natural killer cells (NK). NK cells belong to the innate immune system and play a major role in defending the host from cancer cells, bacteria, and virus-infected cells (Spiering, 2015).

T cells and secreted cytokines are associated with adaptive or cell-mediated immune responses, while B cells and antibodies are key factors in the humoral immune response (Raj and Gothandam, 2015). Immune system imbalances are associated with autoimmune diseases and diseases caused by inflammatory responses in various organs (Shin *et al.*, 2018).

Cyclophosphamide (CP), a commonly used alkylating agent in chemotherapy, has a broad spectrum of activities such as bone marrow suppression, immunosuppression, and cytotoxic effects in a variety of diseases and, recently it has been widely used in immunosuppressed animal models for weight loss, immune cell reduction, and cytokine production suppression (Pass *et al*., 2005; Lee *et al*., 2018; Shin *et al*., 2018; Zhou *et al*., 2018).

In previous studies, high-dose treatment of CP was reported to reduce weight, spleen and thymus weight, total leukocyte count, differential leukocyte, antibodies, bone marrow cells, proliferation of T cells and B cells, and NK cell activity (Hussain *et al.*, 2013).

In particular, administration of CP has been reported to cause immunosuppression by a change in Th1/Th2 ratio (Adams and Hamilton, 1984). In addition, in multiple studies, the major factor contributing to the immunosuppressive effect of CP was reduced proliferation of T cells and the decreased secretion of Th1 cell cytokines (TNF-a, IFN-γ, IL-2, and IL-12) and Th2 cell cytokines (IL-4, IL-6, and IL-10) (Yu *et al.*, 2014; Zhou *et al.*, 2018).

In Korean, red ginseng extract (RGE), a representative immunostrengthening extract, has memory enhancement, inflammatory pain reduction, anti-tumor effects, antioxidant, and immune enhancement effects at 100 - 300 ㎎/㎏ (Sung *et al*., 2000; Lee *et al*., 2008; Lee *et al*., 2017). Especially, the immune-boosting effect of natural materials is compared with RGE in splenocyte from mice and rat (Byun and Byun 2015; Noh *et al.*, 2019). In this study, the efficacy of REG and CZE were also compared.

Recently, the immunomodulatory properties of natural foods and food products have been studied to develop immune enhancers as a component of functional foods, with a wide range of therapeutic properties and relatively low toxicity (Haddad *et al.*, 2005; Chen *et al.*, 2009; Chen *et al.*, 2011). In this study, we investigated the immunostimulatory effect of CZE in isolated splenocytes and CP-induced immunosuppressed rats.

MATERIALS AND METHODS

1. Preparation of *Chrysanthemum zawadskii* Herbich var. *latilobum* Kitamura extracts

C. zawadskii Herbich var. *latilobum* Kitamura (CZ) was provided from BTN Co. Ltd.. Red ginseng powder (GRP) purchased (Daejeon, Korea).

The *C. zawadskii* Herbich var. *latilobum* Kitamura extracts (CZE) and red ginseng extracts (GRE) were extracted in the same protocol.

To prepare CZE and RGE, 100 g CZ and RGP were extracted in an electric boiling pot for 3 h with 1,000 ㎖ of distilled water at 70℃ temperature, and then filtered through a 55 ㎛ bag filter. The solution was evaporated under natural circulation at 55℃ and sterilized at 90℃ for 10 min using a concentrator.

After sterilization, the extract was spray dried under the conditions of electric heater at 227℃, inlet temp 180℃ - 200℃, outlet temp 80℃ - 100°C, nozzle press 40 bar - 70 bar, and then used for each experiment.

2. Animals

Animal experiments were performed as previously described (Lee *et al.*, 2018). Briefly, five-week-old male Sprague-Dawley (SD) (n = 62) rats were purchased from Samtaco Inc. (Osan, Korea) and adapted to the following conditions for 7 days: 12 h light / 12 h dark cycle; temperature, 23 ± 1℃; humidity, 50 ± 5%; and illumination, 150 lux - 300 lux.

The animals were allowed ad libitum access to food (Purina diet; Purina Korea, Seongnam, Korea) and water. Splenocytes were collected from 2 rats for the study. The remaining 60 rats

(10 rats per group) were then randomly assigned to six groups.

SD rats were orally administered CZE (0, 30, 100, or 300 ㎎/㎏/day), RGE (300 ㎎/㎏/day), and CP (5 ㎎/㎏, once per day) for 28 days. Rats treated with saline were used as a normal group.

The protocols used for the animal studies were approved by the Committee on Care and Use of Laboratory Animals of the INVIVO (Nonsan, Korea, Approval No. IV-RA-06-1904-09).

3. Cell culture

Cells were cultured as described previously (Lee *et al.*, 2018). Briefly, the spleen of an 8 week old Sprague-Dawley (SD) rat was aseptically dissected to obtain splenocytes. The spleen was gently pressed with forceps and then forced through a 70 ㎛ cell strainer (SPL Life Sciences Inc., Pocheon, Korea).

The cells were collected and washed three times in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) by centrifugation (80 × g for 3 min, at 4℃). Next, the cells were treated with red blood cell lysis buffer (Sigma-Aldrich Co., St. Louis, MO, USA). Isolated splenocytes were maintained in RPMI-1640 containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin, streptomycin) (Invitrogen, Waltham, MA, USA) in a 5% CO_2 incubator.

4. Cell viability

Cell viability was assessed as previously described (Lee *et al.*, 2018) using a WST-1 assay kit (ITSBio, Seoul, Korea), according to the manufacturer's instructions.

Briefly, splenocytes (2×10^5 cells/well) were seeded into 96-well plates and incubated at 37℃ for 4 h to allow for cell stabilization. Next, the cells were treated with CZE and red ginseng extracts (RGE) (indicated doses in Fig. 2.) and CP (1,600 ㎍/㎖) and incubated for 24 h in a 5% CO_2 incubator.

Each experiment was performed in triplicate. Splenocyte viability rate was assessed using the WST-1 assay kit and a Sunrise™ enzyme-linked immunosorbent assay (ELISA) plate reader (Tecan, Männedorf, Switzerland).

5. NK cell activity assay

NK cell activity was examined as previously described (Lee *et al.*, 2018).

Briefly, AR42J cells obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) were used as target cells for NK cell activity assay, and splenocytes were isolated from control, RGE (positive control) or CZE-treated groups for use as effector cells.

Splenocytes were co-cultured with AR42J cells in 96-well plates at a ratio of effector cells to target cells (25 : 1) and cultured in a 5% CO_2 incubator at 37℃ for 24 h. AR42J viability rate was assessed using the WST-1 Assay Kit and Sunrise™ ELISA plate reader (Tecan, Männedorf, Switzerland).

The NK cell activity was calculated as the survival rate of AR42J compared with that of the control group.

6. Measurement of cytokine levels in splenocytes

This evaluation was performed as previously described (Lee *et al.*, 2018).

Briefly, splenocytes (2×10^5 cells/well) were seeded into 96-well plates with RPMI-1640 containing 10% FBS and 1% antibiotics (growth media), after which CZE (0, 3, 5, 10, 50, 100 and 300 ㎍/㎖), RGE (positive control) and CP (1,600 ㎍/㎖) were added to the wells, and then cells were incubated for 24 h in a 5% CO_2 incubator.

Each experiment was performed in triplicate. The levels of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-2, and IL-12 in the culture medium from each well were then measured using Cytokine Activation Analysis Kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The results were measured using an Sunrise™ ELISA plate reader (Tecan, Männedorf, Switzerland).

7. Complete Blood Count (CBC) and cytokines analyses

These analyses were conducted as previously described (Lee *et al.*, 2018).

Briefly, SD rats were orally administered CZE (0, 30, 100, or 300 ㎎/㎏/day), RGE (300 ㎎/㎏/day), and CP (5 ㎎/㎏, once per day) for 28 days. Rats treated with saline were used as a control group.

After the final administration of the various drugs, the rats were weighed and anesthetized via intraperitoneal injection of 2, 2, 2-tribromoethanol (Sigma-Aldrich Co., St. Louis, MO, USA).

Whole blood was collected through the abdominal vena cava into ethylenediaminetetraacetic acid (EDTA) microcentrifuge tubes. Next, the rats were sacrificed by brief exposure to 100% CO_2, followed by cervical dislocation. The numbers of white blood cells (WBCs), lymphocytes, and granulocytes in each whole blood sample were measured using a Hemavet 950 system (Drew Scientific Group, Dallas, TX, USA).

In addition, mid-range absolute counts (MID), which generally

include monocytes, eosinophils, and basophils, were determined. The plasma levels of TNF-α, IFN-γ, IL-2, and IL-12 were quantified using ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

8. Spleen histochemical analysis

This histochemical analysis was performed as previously described (Lee *et al.*, 2018).

Briefly, after the animals were sacrificed, spleen was removed, weighed, and fixed in 10% neutral buffered formalin. The organs were then processed for embedding in paraffin, after which they were sectioned into 4 ㎛ - 7 ㎛ thick slices using a microtome (Thermo Scientific Inc., Waltham, MA, USA).

The sectioned tissues were then stained with hematoxylin and eosin (H&E). Tissue damage was assessed under an optical microscope (Olympus Co., Fukuoka, Japan).

9. Statistical analysis

Results were analyzed by One-way Analysis of Variance (ANOVA) and Duncan's Multiple Range Tests (DMRT) using SAS software (version 9.3; SAS Institute Inc., Cary, NC, USA). p values < 0.05 were considered statistically significant.

RESULTS

1. Effect of CZE and RGE on CP-mediated reduced splenocytes viability

In order to find out the immune-boosting effect of CZE, an experiment was conducted using RGE, which is well known for immune-boosting with positive control (Byun and Byun, 2015). RGE was extracted in the same manner as CZE and experimented with the same concentration.

To examine any cytotoxic effects of CZE and RGE on splenocytes, cells were incubated with different concentrations of CZE and RGE (0, 3, 5, 10, 30, 50, 100, 300, 500, 1,000 and 3,000 ㎍/㎖) for 24 h. Splenocytes did not show a change in cell viability at concentrations bellow 500 ㎍/㎖ of CZE and 1,000 ㎍/㎖ of RGE (Fig. 1A).

To investigate cell proliferation, splenocytes were incubated with different concentrations of CZE and RGE (0, 3, 5, 10, 30,

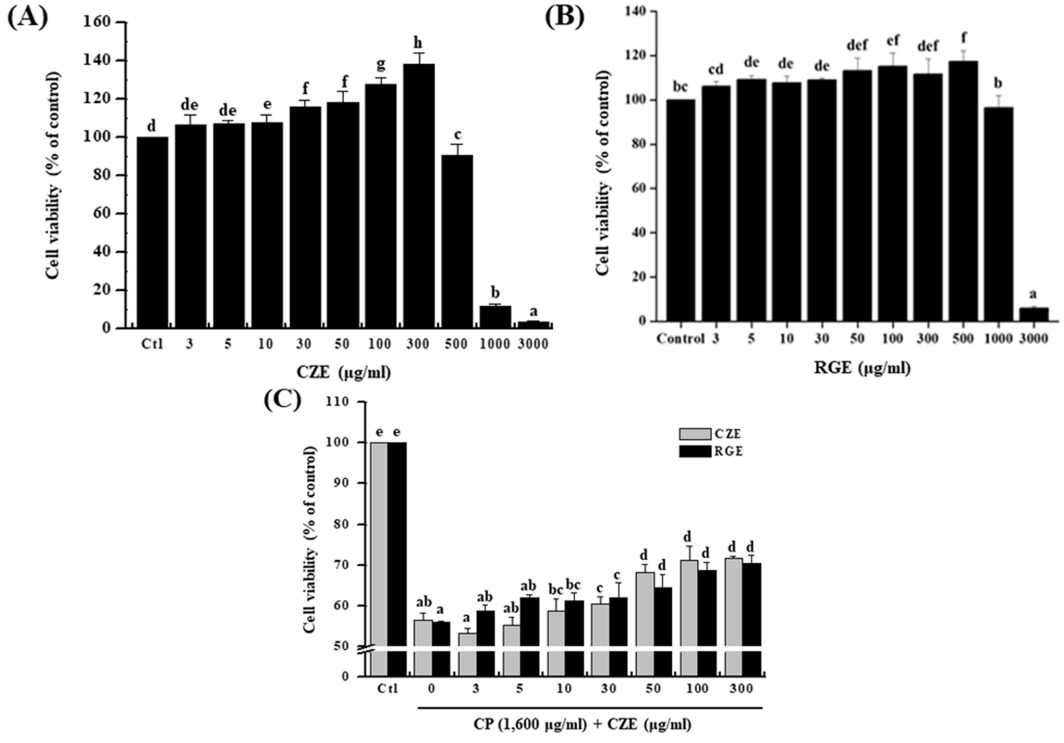

Fig. 1. Effect of *Chrysanthemum zawadskii* Herbich var. *latilobum* Kitamura extracts (CZE) and red ginseng extract (RGE) on the viability and cyclophosphamide (CP)-stimulated cell proliferation in splenocytes from SD rats. (A and B) Splenocytes were seeded into a 96-well plate with CZE (0, 3, 5, 10, 30, 50, 100, 300, 500, 1,000 and 3,000 ㎍/㎖). (C) Splenocytes were treated with CZE (0, 3, 5, 10, 30, 50, 100 and 300 ㎍/㎖) or/and CP (1,600 ㎍/㎖) for 24 h in a 5% CO_2 incubator, and then cell viability rates were measured with WST-1 Assay Kit. Data are presented as means ± standard errors (n = 3). Bars labeled with different superscripts indicate significant differences (p < 0.05 versus control) by Duncan's Multiple Range Test (DMRT).

50, 100 and 300 ㎍/㎖) and/or CP (1,600 ㎍/㎖) for 24 h. CZE and RGE increased the cell proliferation in a dose-dependent manner (Fig. 1B), suggesting that CZE and RGE could recover from the reduced cell proliferation by CP.

2. Effect of CZE on CP-induced reduced expression of cytokine in splenocytes

The immune-boosting effect of CZE in CP-reduced expression of cytokines was investigated. Splenocytes from SD rat was incubated with CZE (0, 3, 5, 10, 50, 100 and 300 ㎍/㎖), RGE (positive control) and CP (1,600 ㎍/㎖) for 24 h. CZE and RGE increased the levels of TNF-α, IL-2, and IL-12 in a dose-dependent manner (Fig. 2).

Our results showed that CZE-treatment increased the CP-mediated reduction of levels of TNF-α, IL-2, and IL-12. The level of IFN-γ tends to increased CZE and RGE, but it was not significant difference compared to control. These result explained that CZE has a similar effect to RGE, which has an immune-boosting effect.

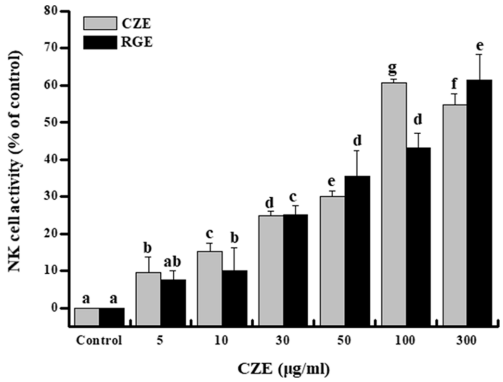

Fig. 3. Effect of CZE on splenic NK cell activity in splenocytes from SD rats. NK cell activity assayed by the WST-1 assay as described in the text. Isolated splenocytes were co-cultured with target cells (AR42J) for NK cell activity in 96-well plates, followed by treatment with CZE (0, 5, 10, 30, 50, 100 and 300 μg/㎖) and incubated for 24 h in a 5% CO_2 incubator with a ratio of effector to target cells of 25:1. The NK cell activities was calculated as the survival rate of AR42J compared to that of the control group. Data are presented as means ± standard errors (n = 3). Bars labeled with different superscripts indicate significant differences ($p < 0.05$ versus control) by Duncan's Multiple Range Test (DMRT).

3. Effects of CZE on NK cell activity in CP-treated splenocytes

NK cells play important roles in defense against virus-infected cells and tumor cells (Medzhitov and Janeway, 1997).

Therefore, we confirmed the effects of CZE and RGE (positive control) on NK cell activity. Splenocyte cytotoxicity

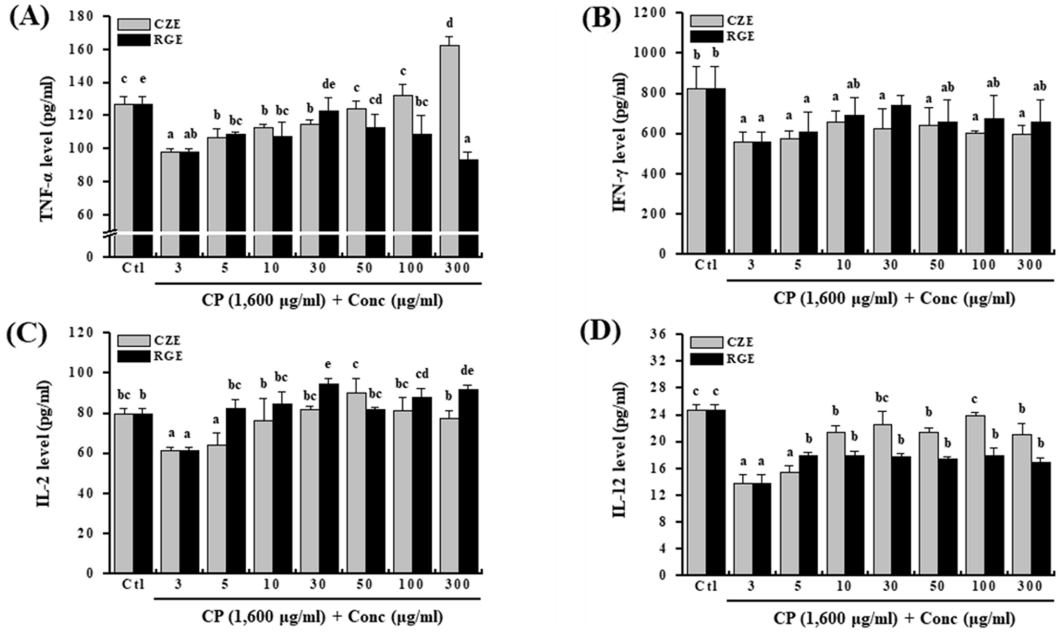

Fig. 2. Effect of CZE on cytokine levels in splenocytes from SD rats. Isolated splenocytes were seeded into 96-well plates, followed by treatment with CZE (0, 3, 5, 10, 30, 50, 100 and 300 μg/㎖) and/or CP (1,600 μg/㎖) and incubated for 24 h in a 5% CO_2 incubator. Levels of cytokines (TNF-α, IFN-γ, IL-2, and IL-12) of culture medium were analyzed using ELISA kits. Data are presented as means ± standard errors (n = 3). Bars labeled with different superscripts indicate significant differences ($p < 0.05$ versus control) by Duncan's Multiple Range Test (DMRT).

was tested against NK cell-sensitive tumor cells (AR42J). As shown in Fig. 3, CZE and RGE treatment resulted in increased NK cell activity in a dose-dependent manner. CZE significantly increased from 5 ㎍/㎖. And RGE significantly increased from 10 ㎍/㎖.

These results show that CZE was increased at a concentration lower than RGE, and explained that CZE could improve the cell immune response in SD rats.

4. Effect of CZE on numbers of immune cells in CP-induced immunosuppressed rats

CP is known to reduce viability of immune cells. Immune cells were measured in blood of CP (5 ㎎/㎏/day), CZE (intake 30, 100, or 300 ㎎/㎏/day for 28 days) with CP, and RGE (intake 300 ㎎/㎏/day for 28 days). Concentration of RGE was a positive group, which has similar results to CZE in Fig. 1 – Fig. 3, and was set to the same concentration as the high-concentration of CZE (300 ㎎/㎏/day).

Concentration-dependent of CZE increased the number of WBCs, lymphocytes, MID (monocyte, eosinophil, and basophil), and granulocytes in CP-induced immunosuppressed rats (Fig. 4).

5. Effect of CZE on serum levels of cytokines in CP-induced immunosuppressed rats

Next, we confirmed the effects of CZE on the serum levels of immune-related cytokines such as TNF-α, IFN-γ, IL-2, and IL-12 in CZE and RGE (positive control)-treated rats and/or CP-induced immunosuppressed rats (Fig. 5).

The serum levels of cytokines were lower in CP-induced immunosuppressed rats compared with that in normal rats (saline-treated). However, CZE-treated groups showed an increase in plasma levels of TNF-α, IL-12 and IFN-γ compared with the non-CZE treated group, but not significantly increased IL-2 levels. Th1 cell promoting factors include IL-12 and IFN-γ. And Th1 cells are source for the inflammatory cytokines such as lFNγ, IL-2, and TNF-β. Therefore, these results

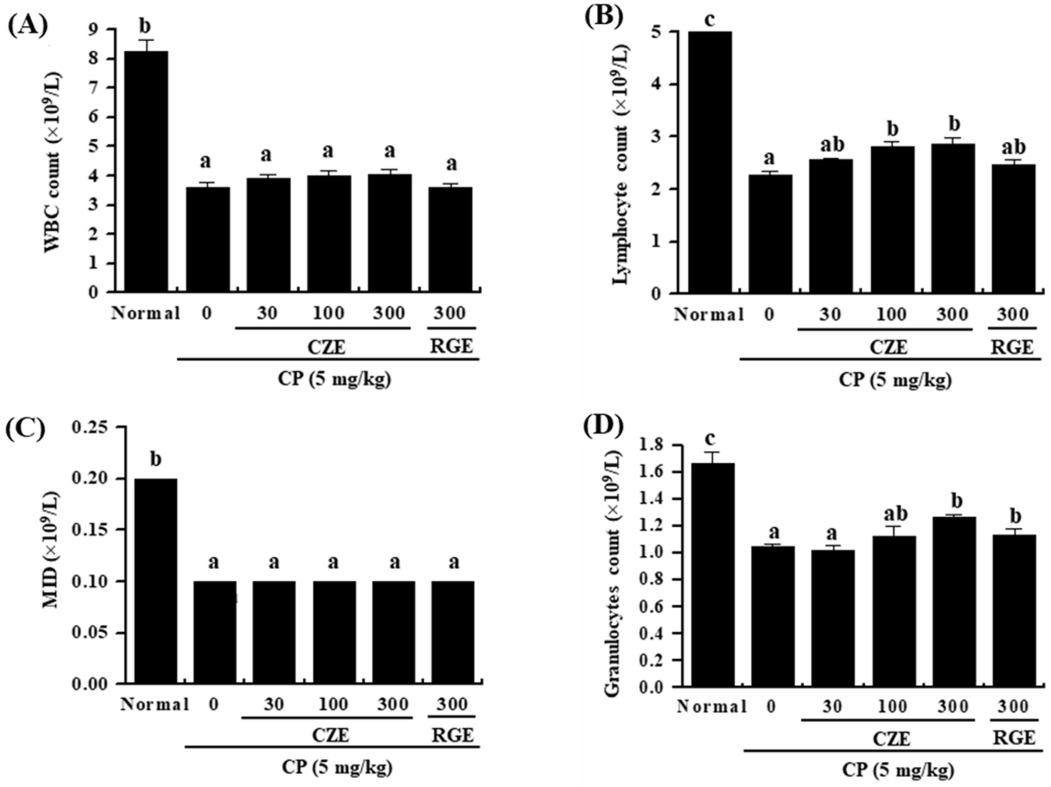

Fig. 4. Effects of CZE on inflammatory cells counts in the whole blood of CP-induced immunosuppressed rats. SD rats were administrated with saline, CP (5 ㎎/㎏/day), and CZE (0, 30, 100, or 300 ㎎/㎏/day) or RGE (300 ㎎/㎏/day) once daily for 28 days, after which whole blood samples were collected. The levels of inflammatory cells (WBCs, lymphocytes, MID, and granulocytes) in the blood samples were determined using a Hemavet 950 system. Data are presented as means ± standard errors (n = 3). Bars labeled with different superscripts indicate significant differences ($p < 0.05$ versus control) by Duncan's Multiple Range Test (DMRT).

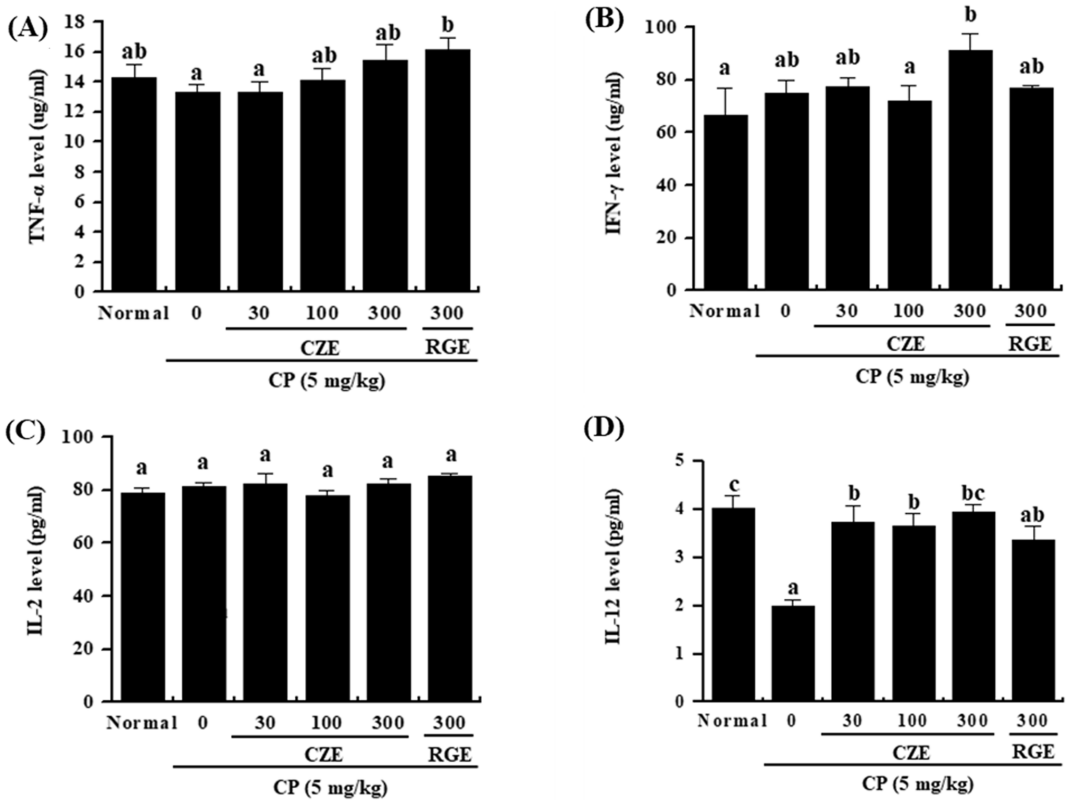

Fig. 5. Effect of CZE on the levels of cytokines in the serum of CP-induced immunosuppressed rats. SD rats were administrated with saline, CP (5 ㎎/㎏/day), and CZE (0, 30, 100, or 300 ㎎/㎏/day) or RGE (300 ㎎/㎏/day) once daily for 28 days, after which serum levels of TNF-α, IFN-γ, IL-2, and IL-12 were quantified using ELISA kits. Data are presented as means ± standard errors (n = 7). Bars labeled with different superscripts indicate significant differences ($p < 0.05$ versus control) by Duncan's Multiple Range Test (DMRT).

indicate that CZE improved the reduced Th1 cytokine production in CP-induced immunosuppressed rats.

6. Effect of CZE on spleen morphology in immunosuppressed rats

The morphological changes in the spleen were investigated after CZE and RGE (positive control) treatment using H&E staining. Spleen confirmed that 30 ㎎/㎏, 100 ㎎/㎏, and 300 ㎎/㎏ of CZE gradually promoted the spleen cell multiplication of white pulp compared with the CP-treated rats (Fig. 6).

In the spleen tissue of the normal group, the red pule (RP) was clearly observed as the white pule (WP) was evenly distributed around central vein and the LN (lymph nodle) was surrounded by the marginal zone (MZ). However, in the control group (only CP), disruption of WP and condenstation of cells in RP were observed.

In the spleen tissue of the CZE treated groups, tissue collapsed by CP did not improve in CZE (30 ㎎/㎏) treated group, it showed an improvement from CZE (100 ㎎/㎏) treated group, and at high concentration (300 ㎎/㎏) treated group, it improved more than the positive control group (RGE. 300 ㎎/㎏).

This result suggested that CZE stimulated innate and adaptive immunity by ameliorating the CP-mediated spleen tissue damage.

DISCUSSION

Chrysanthemums are perennial flowering plants in the Compositae family which is native to Asia and northeastern Europe. *Chrysanthemum zawadskii* Herbich var. latilobum is one of the species of the genus Chrysanthemum and has traditionally been used in folk medicine, known as ‘Gujeolcho’ in Korea for the treatment of various diseases. *C. zawadskii* Herbich var. latilobum extracts has been shown to have anti-inflammatory and anti-oxidative stress activities in RAW 264.7 murine macrophage cells (Han *et al.*, 2002).

In previous study showed that the extract of *C. zawadskii*

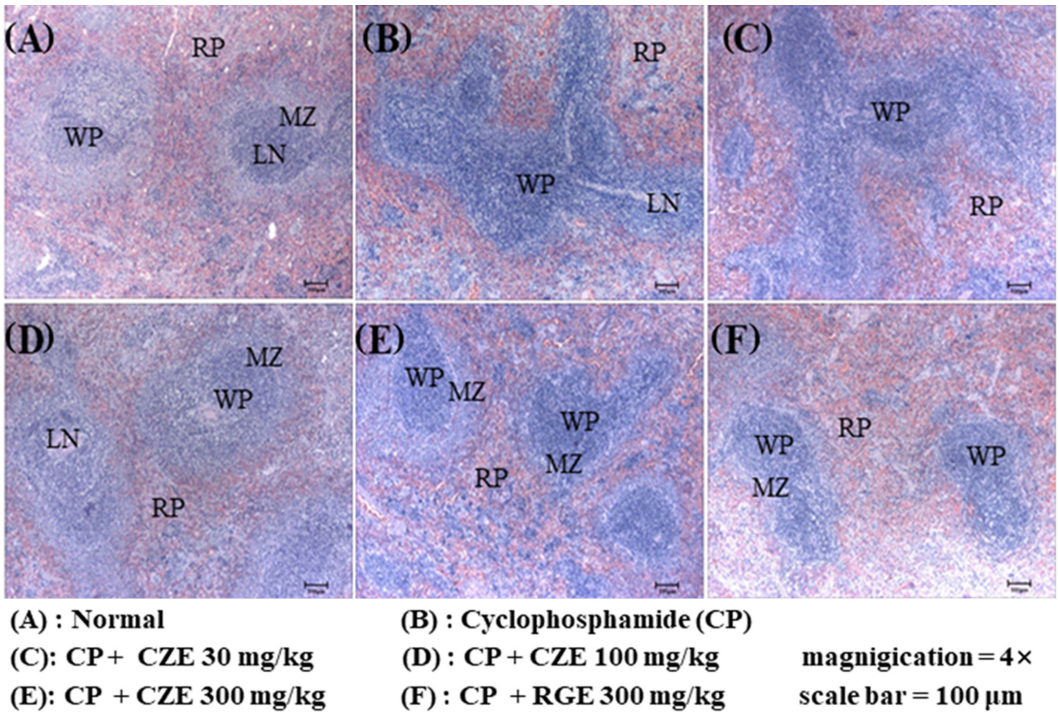

Fig. 6. Effect of CZE on immunity-associated spleen damage in CP-induced immunosuppressed rats. SD rats were administrated with saline, CP (5 ㎎/㎏/day), and CZE (0, 30, 100, or 300 ㎎/㎏/day) or RGE (300 ㎎/㎏/day) once daily for 28 days, after which damage of spleen was analyzed histologically. Representative images of the sectioned spleens of (A); normal rats (saline treatment), (B); control rats (treated with only CP), (C - E); CP and CZE-treated rats [(C); 30 ㎎/㎏, (D); 100 ㎎/㎏, or (E); 300 ㎎/㎏ CZE] and (F); CP and RGE-treated rats. Scale bar = 100 ㎛. WP; white pulp, LN; lymph nodule, MZ; marginal zone, RP; red pulp.

strongly suppressed BMM-derived osteoclast differentiation by regulate NF-κB pathway (Gu *et al.*, 2013). Previous studies have explained that through NF-κB pathway has anti-inflammatory effects. And it is reported that extracts with anti-inflammatory effects have immune-boosting effects (Kim *et al.*, 2021). CZE was also thought to have an immune-boosting effect.

Recent studies have been actively conducted to confirm the immunostimulatory effects of natural products and food ingredients (Fang *et al.*, 2015; Wang *et al.*, 2015; Lee *et al.*, 2016; Song *et al.*, 2017). In the current study, we demonstrated that CZE ameliorated the CP-mediated immunosuppression.

Our results shown that CZE treatment restored CP-mediated reduced cell proliferation, cytokines level (such as TNF-α, IL-2, IL-12 and IFN-γ), and NK cell activity in splenocytes from SD rats. Moreover, CZE treatment also improved blood levels of immune cells, cytokines (TNF-α, IL-12 and IFN-γ) and CP-induces spleen damage improved upon treatment with CZE in immunosuppressed animal models.

CP is generally used as an important chemotherapeutic drug in tumor therapy; however, it is known to sometimes cause side effects such as bone marrow suppression, immunosuppression, and oxidative stress (Wang *et al.*, 2011).

In many studies, CP-treated rats have been used as immunosuppressed animal models (Lee *et al.*, 2018; Wang *et al.*, 2018; Yan *et al.*, 2021). Immunosuppression by CP was reported to significantly reduce RBC, WBC, and platelet levels, inhibit spleen NK cell and CTL activity, decrease CD4 T lymphocyte count and CD4/CD8 ratio (Diwanay *et al.*, 2004, Huang *et al.*, 2007; Yan *et al.*, 2021).

In addition, CP decreases the levels of serum cytokines (IL-2, IFN-γ, and IL-10) (Huang *et al.*, 2007, Hu *et al.*, 2009). CP damages the spleen, which are important for the immune response (Li *et al.*, 2017).

In our study, we investigated the effect of CZE on various immunoregulatory markers in CP-induced immunosuppressed rats.

Proliferation of lymphocytes and monocyte/macrophage is important for the activation of humoral and cellular immune responses. Splenic cells are composed of various immune cells

such as T and B cells, macrophages and dendritic cells (Klimp *et al.*, 2002). In a recent studies, it is reported that proliferation of splenocytes decrease the survival rate by CP.

An immunosuppressive agent, CP reduced the proliferation of splenocytes, it explains the reduction of immunity (Lee *et al*., 2018). On the other hands, splenocyte proliferation ultimately enhances immunity through increased cell mediated immune response by the expression of cytokines (Zeng *et al.*, 2013; Conniot *et al.*, 2014).

In our results, CZE increased the immune cell survival rate, and suppressed the CP-mediated loss of cell viability (Fig. 1B and 4).

The cytokines expressed by various immune cells play an important role in immune responses such as host defense against bacterial infection, cell survival, apoptotic lymphocyte differentiation, and inflammation regulation (Aggarwal, 2003; Lin *et al.*, 2007; Boyman and Sprent, 2012).

Several types of cytokines secreted by Th1 and Th2 cells are the determinants of cell function. Th1 cells secrete IL-2, TNF-α, and IFN-γ, which are important cytokines in the humoral immune response (Tough *et al.*, 1997; Ulmer *et al.*, 2000; McAleer and Vella, 2008), while Th2 cells mainly secrete IL-4, IL-6, and IL-10 to induce cell mediated immune responses (Decker *et al.*, 2005).

Our study indicated that CZE plays an important role in inducing Th1 cytokine secretion and subsequent immune response in the immunosuppressed state, confirming the immunostimulatory effect of CZE (Fig. 2 and 5).

NK cells, cytotoxic lymphoid cells play a major role in the initial immune response stage to remove foreign, abnormal cells, viaral infcted and tumor cells (Kos and Engleman, 1996; Medzhitov and Janeway, 1997; Pierce *et al.*, 2020). NK cells are activated by stimuli of cytokines and chemokines and are known to play a central role in regulating tumor growth and metastasis and removing viruses (Kos and Engleman, 1996; Sarangi *et al.*, 2006). Therefore, measurement of NK cell is a useful method for assessing the cellular immune response of the host (Elemans *et al.*, 2011).

Our results showed that spleen NK cell activity was significantly increased by CZE treatment (Fig. 3).

The immune system is weakened by birth, aging, disease, or a variety of other causes (Motta M *et al.*, 2007). In our previous study, decreased immunity has resulted in a decrease in the number of white blood cells, NK cells, and immune-related cells in rat. Therefore, these cells play an important role in maintaining immunity (Lee *et al.,* 2007).

In Fig. 4, we showed that CP-induced immunosuppression reduced WBC, lymphocyte, MID (monocytes, eosinophils, and eukaryotes) and granulocytes, consistent with other reports (Davis and Kuttan, 1999, Shalit *et al.*, 2001). However, CZE administration could significantly restore the number of immune cells in CP-treated rats. In addition, it was confirmed that the CP-induced damage (disruption of WP) of spleen was restored to the normal state by CZE (Fig. 6). These results suggest that CZE improves immune response by restoring CP-induced impaired immune system function in spleen.

In conclusion, the results of this study confirm that CZE help innate and adaptive immune processes by increasing levels of cytokines, NK cell activity, and immune cell production under immunosuppressive conditions. These results suggest that CZ could be used as a material for the development of functional food for immunity enhancement.

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