KOREAN J. FOOD SCI. TECHNOL. Vol. 51, No. 4, pp. 398~403 (2019)
https://doi.org/10.9721/KJFST.2019.51.4.398

KOREAN JOURNAL OF
한국식품과학회지
FOOD SCIENCE AND TECHNOLOGY

Enhancement of immune activities of *Dioscorea japonica* Thunberg in *in vivo* and *ex vivo* models

Seokwon Lim[1,†], Na-Hyung Kim[2,†], Hi-Jae Cho[3], and Hyun-Ja Jeong[1,*]

[1]*Division of Food and Pharmaceutical Engineering*
[2]*Department of Pharmacology, College of Korean Medicine, Kyung Hee University*
[3]*Dongsan Natural Products Co.*

Abstract *Dioscorea japonica* Thunberg (DJ) has been widely used as a healthy food in Korea for the enhancement of physical stamina. Hence, the present study evaluated the immune-enhancing effect of DJ in forced swim test of mouse model. The immobility time of the group treated with DJ for 7 days was significantly reduced in comparison with that of the control group. After a forced swimming test, the changes in blood biochemical parameters and splenic T lymphocyte populations induced by the administration of DJ were assessed. Serum levels of lactic dehydrogenase, creatine phosphokinase, and aspartate aminotransferase were significantly decreased in DJ-administered group compared to the control group. However, administration of DJ did not affect the splenic T lymphocyte populations. Moreover, DJ significantly increased the production of interferon-g and interleukin-2 compared to the media control in splenocytes. Collectively, it may be concluded that DJ is useful for enhancement of physical and immune function.

Keywords: *Dioscorea japonica* Thunberg, forced swimming test, interferon-g, interleukin-2, immune-enhancement

Introduction

The forced swimming test is commonly used as a screening test for anti-fatigue properties of drugs or health functional food and also as test to examine immune-enhancing effects of candidate agents (Kim et al., 2016; Nam et al., 2016). It has been reported that forced-swimming test exposure produces alterations in both cellular and noncellular immunity (Connor et al., 1997). Forced-swimming test exposure causes a reduction in the proliferation of lymphocytes and production of interleukin (IL)-2 and an increase in the percentage of neutrophils in the peripheral blood (Connor et al., 1997).

An immune system consists of two immune responses by innate immune signaling and adaptive immune signaling. Innate immune system involves innate cells such as dendritic cells, natural killer cells, mast cells, and macrophages to produce anti-inflammatory molecules such as IL-10 and transforming growth factor-α, and adaptive immune system mainly generates lymphocytes such as T cells which can directly destroy virus-infected cells or release cytokines (Rouse and Sehrawat, 2010). T cells play a crucial role in immune functions as they act both as effectors (cytotoxic T cells, $CD8^+$) and regulators (helper T (Th) cells, $CD4^+$). Th cells mediate the link between the antigen-presenting and triggering of other cellular and humoral components of the immune-response (Riddell et al., 2002). Th cells have two different subsets, Th1 and Th2. The ratio of Th1 to Th2 is correlated with the outcome of many diseases and controlling this ratio has been utilized as a therapeutic strategy for various immune related diseases. The induction of Th1 lymphocytes, which produce interferon (IFN)-γ, IL-2, and tumor necrosis factor, in the immune response plays a critical role in protecting against various intracellular microorganisms and tumors, and is involved in reversing Th2 cells, which produce IL-4-, IL-5-, IL-10- and IL-13-facilitating diseases that include types of allergic inflammation (Cohn and Ray, 2000; Stephens et al., 2002). These cytokines play an important role in the immune response.

Dioscorea japonica Thunberg (DJ, Chamma) has been reported that it contains a group of storage proteins and has anti-oxidative, cholesterol-lowing, and anti-cholinerasterase activities (Hou et al., 2001; Hou et al., 2002; Lee et al., 2006; Oh et al., 2004). One of the components in Dioscorea batatas, dioscorin, which consists of polypeptides of 31 kDa and is a carbonic anhydrase, exhibits antioxidant, anti-allergic effect, radical scavenging activity, and biological activities which could contribute to resistance to pests, pathogens or abiotic stresses (Oh and Lim, 2009; Shewry, 2003). However, it is not clear that DJ regulates the expression of the cytokines in immune system as an aspect of enhancing immunity. Therefore, the present study was designed to investigate the immune enhancing effect of DJ on the immobility time, blood biochemical parameters, and splenic T lymphocyte populations in forced swimming test animal model. Furthermore, we investigated the effect of DJ on cell viability and release of cytokines including interferon (IFN)-γ, interleukin (IL)-2, and IL-4 in splenocytes.

[†]These authors contributed equally to this work.

*Corresponding author: Hyun-Ja Jeong, Division of Food and Pharmaceutical Engineering, Hoseo University, 20, Asan, Chungnam 31499, Republic of Korea
Tel: +82-41-540-9681
Fax: +82-41-542-9681
E-mail: hjjeong@hoseo.edu
Received July 4, 2019; revised July 30, 2019;
accepted July 30, 2019

Materials and Methods

Chemicals

Roswell Park Memorial Institute medium (RPMI) 1640, ampicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Monoclonal antibodies used for flow cytometric analyses of splenic subsets were fluorochrome-conjugated anti-CD4 and anti-CD8 from BD Pharmingen (San Jose, CA, USA). Dimethyl sulfoxide (DMSO), concanavaline A (ConA), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), avidine-peroxidase, bovine serum albumin (BSA), and 2-AZINO-bis (3-ethylbenzi-thiazoline-sullfonic acid) tablets substrate (ABTS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-mouse IFN-γ, IL-2, and IL-4 monoclonal antibodies, biotinylated anti-mouse IFN-γ, IL-2, and IL-4 and recombinant IFN-γ, IL-2, and IL-4 were purchased from R&D Systems (Minneapolis, MN, USA).

Preparation of DJ

DJ used in this study was obtained from an oriental drug store, Noa Pharmacy (Seoul, Korea). DJ was prepared in the form of dried extracts for the *in vitro* study and as dried extract form or crude liquid form for the *in vivo* study. A preparation of form of dried extract of DJ was prepared by decocting the dried DJ with boiling distilled water (100 g/L). The duration of decoction was about 3 h. The decoction was filtered, lyophilized and kept at 4°C. The yield of dried extract from initial crude materials for the *in vitro* and the *in vivo* study was approximately 5.1%. After the samples were dissolved in saline, they were then filtered through a 0.45 μm syringe filter and kept at 4°C. For the *in vivo* study as a crude liquid form, DJ were used after press the juice, dilute in distilled water at a ratio of 1:1, and agitate. We obtained the liquid extraction of 80 mL out of DJ of 100 g. The DJ or saline, respectively, was orally administered into mice, 10 mL/kg, once per day for 7 days using a feeding atraumatic needle according to previous study (Shin et al., 2006).

Animals

Male balb/c mice (5-week-old, 19-21 g) were purchased from the Dae-Han Experimental Animal Center (Daejon, Korea), and then maintained at the College of Oriental Medicine, Kyung Hee University. Mice were housed under following laboratory conditions: temperature 22±1°C, humidity 55±10%, 12:12-L/D cycle, and lights on at 07:00 h. Food and water were available *ad libitum*. All protocols were approved by the institutional animal care and use committee of Kyung Hee University [KHUASP (SE)-10-032]. All manipulations were carried out between 09:00 and 16:00 h, and no animal was used more than once.

Evaluation of forced swimming test

After the first measurement of the immobility time, the mice (n=5/group) were divided into the control group and DJ groups to match the swimming time in each group. DJ was administered into mice once per day for 7 days. FST was conducted at the end of the 7 days administration period. During the 6 min of the forced swimming test, the duration of immobility was measured as previously described by Porsolt et al. (1977). The apparatus consisted of two Plexiglas cylinders (height: 25 cm, diameter: 10 cm) placed side by side in a Makrolon cage filled with water (10 cm height) at 23-25°C. Two mice were tested simultaneously for a 6 min period inside vertical Plexiglas cylinders; a nontransparent screen placed between the two cylinders prevented the mice from seeing each other. The total duration of immobility, after a delay of 2 min, was measured during a period of 4 min. Each mouse was considered to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water.

Analysis of blood biochemical parameters

After the last forced swimming test, mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (4 mg/kg). Blood (1 mL) was withdrawn from the heart of the mice into syringes. Then, serum was prepared by centrifugation at 1500×g, 4°C for 10 min. Contents of glucose, total protein, albumin, lactate dehydrogenase (LDH), blood urea nitrogen (BUN), creatine phosphokinase (CPK), and aspartate aminotransferase (AST) were determined by an autoanalyzer (Hitachi 747, Hitachi, Tokyo, Japan).

Flow cytometry analysis

Spleen was separated immediately after blood sampling and spleen cells were obtained by sieving the tissue through a nylon filter and resuspending the resulting cells in phosphate buffered saline (PBS) solution with 1% BSA and 0.05% sodium azide. Single cell suspensions were incubated with fluorescence-conjugated antibodies, CD4-phycoerythrin (PE) and anti-CD8-peridinin chlorophyll protein (PerCP), at 4°C for 30 min. After incubation, the stained cells were washed and analyzed by a fluorescence activated cell sorter (FACSCalibur) flow cytometer equipped with CELLQuest software (BD bioscience, San Jose, USA). The markers for determining positive and negative cells were set according to negative controls in all cases to account for background fluorescence. The CD4:CD8 ratio was calculated by dividing % $CD4^+CD8^-$ cells by % $CD4^-CD8^+$ cells.

Splenocytes culture

The spleen (balb/c mice, 5-week-old, 19-21 g) was removed aseptically and teased into a single cell suspension in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U/mL), streptomycin (100 mg/mL), and 50 mM 2-mercaptoethanol (Sigma Chemical Co.). Red blood cells were removed by lysis with 0.14 M Tris-buffered NH_4Cl. The remaining cells were washed twice with culture medium. The cells were then cultured in complete medium at 3×10^5 cells per well in a 96-well flat-bottom culture plate. Cells were stimulated with DJ as a final concentration of 1 mg/mL and 10 mg/mL for 24 h in the absence or presence of ConA (2 mg/mL) at 37°C in 5% CO_2. Supernatants were harvested after 24 h for a quantification of cytokine levels.

Cell viability assay

Cell aliquots (3×10^5 cells/well) were seeded in microplate wells and incubated with 20 mL of a MTT solution (5 mg/mL) for 4 h at 37°C under 5% CO_2 and 95% air. Consecutively, 1 mL of DMSO was added to extract the MTT formazan and the absorbance of each well was read at 540 nm by an automatic microplate reader (Molecular Devices, San Jose, CA, USA).

Cytokine assay

A sandwich enzyme-linked immunosorbent assay (ELISA) was carried out for IFN-γ, IL-2, and IL-4 in duplicate using a 96-well format. ELISA plates (Nunc, Denmark) were each coated with 100 mL aliquots of anti-mouse IFN-γ, IL-2, and IL-4 monoclonal antibodies at 1 mg/mL in PBS at pH 7.4 and were then incubated overnight at 4°C. The plates were washed in PBS containing 0.05% tween-20 and blocked with PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3 for 1 h. After additional washes, 100 mL sample or each standard was added and the plates were then incubated at 37°C for 2 h. After 2 h incubation at 37°C, the wells were washed and then each of 0.2 mg/mL of biotinylated anti-mouse IFN-γ, IL-2, and IL-4 were then added to each and they were incubated again at 37°C for 2 h. After washing the wells, avidin-peroxidase was added and plates were incubated for 30 min at 37°C. Wells were again washed and ABTS substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader. A standard curve was run on each assay plate using recombinant IFN-γ, IL-2, and IL-4 in serial dilutions.

Statistical analysis

Results were expressed as the mean±SD of independent experiments, and statistical analyses were performed by a one-way analysis of variance (ANOVA) with Tukey's, and Duncan post hoc tests to express the differences among the groups. All statistical analyses were performed using SPSS v12.0 statistical analysis software (IBM Corporation, Armonk, NY, USA). A value of $p<0.05$ was considered to indicate statistical significance.

Results and Discussion

Effect of DJ on immobility time of forced swimming test

Swimming can be used effectively as a stress factor since mice are not normally aquatic, and it is accepted as an experimental exercise model, the forced swimming test also is one of the most commonly used behavioral tests for rodents (Hilakivi-Clarke and Dickson, 1995). The swimming test causes alterations in cellular and non-cellular immunity, lowers the ratio of lymphocytes and enhances the ratio of neutrophils in rat peripheral blood (Connor et al., 1997; Delbende et al., 1994; Dubovik et al., 1987). Recently, the forced swimming test has been used to examine whether a certain agent has immune-enhancing, anti-fatigue, and anti-depressant effect (Han et al., 2018; Kim et al., 2016). So, we performed the forced swimming test to assess immune-enhancing effect of DJ. The mice were divided into a control group, a dried extract form of DJ-administered group, and a crude liquid form of DJ-administered group. Using the forced swimming test, the immobility times in the respective three groups were measured after administration for 7 days. As a result, the immobility time was significantly decreased in the DJ-administered groups compared with the control group ($p<0.05$, Fig. 1). Therefore, this result suggests that DJ has an immune-enhancing effect.

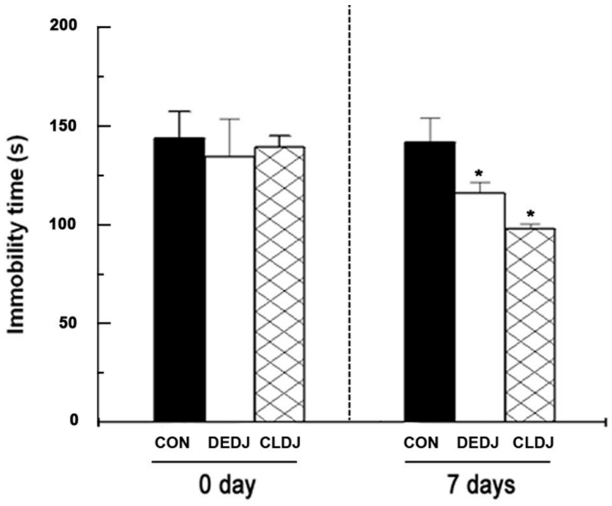

Fig. 1. Effect of DJ on immobility time of the mouse forced swimming test. The administration of DJ was continued for 7 days at the same time. On the forced swimming test, the administration of DJ was conducted 1 h before the test. Values are means±SD. *$p<0.05$ *vs.* saline-administered control group. CON, a saline-administered control group; DEDJ, a dried extract form of DJ (10 mg/kg/day, *p.o.*)-administered group; CLDJ, a crude liquid form of DJ (10 mL/kg/day, *p.o.*)-administered group.

Effect of DJ on blood biochemical parameters

It is known that the swimming exercise induce biochemical changes in blood (De-Mello, 1992). The serum glucose levels were generally decreased immediately after the forced swimming test (Rose and Sampson, 1982). Levels of total protein and albumin can reflect the nutritional state, kidney disease, and chronic liver disease, and can also indicate the condition between the extracellular and intracellular fluids (Costill and Fink, 1974). The muscle produces plenty of lactic acid, when it obtains enough energy from anaerobic glycolysis at nearly the same time as the high-intensity exercise. The increased lactic acid levels will bring about a reduction of pH in muscle tissue and blood, and also induce many side effects of various biochemical and physiological processes (Okazaki et al., 1996). BUN and creatinine are standard metrics used to diagnose and monitor kidney injury (Ferquson et al., 2008). CPK is present in both skeletal and cardiac muscle and is released into the blood when myocyte necrosis occurs (Van der Veen and Willebrands, 1966). AST is normally found in red blood cells, liver, heart, muscle tissue, pancreas, and kidneys, and catalyze the reductive transfer of an amino group from aspartate. The amount of AST in the blood is directly related to the extent of the tissue damage. After severe damage, AST levels rise in 6 to 10 hours and remain high for about 4 days. Serum AST activity is considered a less specific biomarker of liver function and is released from damaged myocytes as well as hepatocytes (Ozer et

Table 1. Body and spleen weight and blood biochemical parameters value

	CON	DEDJ	CLDJ
Body weights			
Initial (g)	20.3±0.92	20.1±1.17	20.3±0.99
Final (g)	23.0±0.59	23.7±0.58	23.6±0.74
Spleen weights (mg)	88.0±4.47	86.0±5.48	84.0±8.94
Blood parameters			
Glucose (mg/dL)	217.0±23.26	240.3±9.17	222.0±23.07
Protein (g/dL)	5.5±0.15	5.6±0.01	5.2±0.09
Albumin (g/dL)	3.4±0.09	3.4±0.03	3.1±0.09
LDH (IU/L)	839.0±58.13	603.7±50.81*	529.0±25.79**
BUN (mg/dL)	28.6±1.52	23.4±1.30	23.8±2.98
CPK (mg/dL)	397.3±47.64	262.7±86.90	162.3±42.30*
AST (IU/L)	103.0±6.81	84.3±7.27	75.7±2.91*

CON; a saline-administered control group, DEDJ; a dried extract form of DJ-administered group, CLDJ; a crude liquid form of DJ-administered group. Values represent the mean±SD.
*p<0.05, **p<0.01: significantly different from CON.

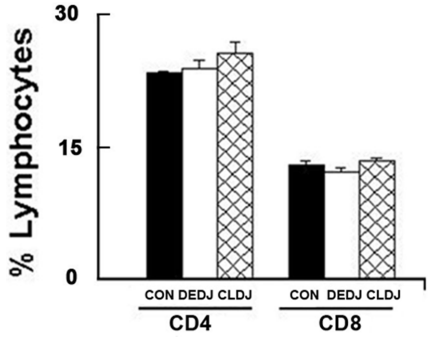

Fig. 2. Effect of DJ on T cell population of spleen after forced swimming test. Percentages of T cell subsets. Splenocytes stained with anti-CD4-phycoerythrin (PE) and anti-CD8-peridinin chlorophyll protein (PerCP) cell surface marker antibodies and then analyzed by flow cytometry. Each result was obtained from five mice per group and is representative of three experiments. Each datum represents the mean±SD. CON, a saline-administered control group; DEDJ, a dried extract form of DJ (10 mg/kg/day, *p.o.*)-administered group; CLDJ, a crude liquid form of DJ (10 mL/kg/day, *p.o.*)-administered group.

al., 2008). As is commonly known, exercise or stress increases the serum concentrations of LDH, BUN, CPK, and AST (Ferquson et al., 2008; Okazaki et al., 1996; Ozer et al., 2008). In this study, the levels of blood biochemical parameters were assessed in mice after the force swimming test to clarify the mechanism in the reduction of immobility time by DJ. Serum levels of glucose, protein, and albumin were not changed in DJ-administered groups compared with the control group. The LDH levels were significantly decreased in both a dried extract form of DJ-administered group and a crude liquid form of DJ-administered group compared with the control group (p<0.05, Table 1). The DJ-administered groups compared with the control group in the BUN level tended to reduce, but did not show significant differences (Table 1). The serum levels of CPK and AST were significantly reduced by oral administration of a crude liquid form of DJ (p<0.05, Table 1). Therefore, these results suggest that DJ improves the blood biochemical parameters damaged by FST.

In this study, a crude liquid form of DJ-administered group showed more efficacy than a dried extract form of DJ-administered group on blood biochemical parameters. The extraction methods for herbs or materials such as toasting, boiling in honey, steaming, and dipping or soaking in alcohol, water, or vinegar can increase their desirable effect, and decrease toxicities and side effects (Lee et al., 2003). Studies investigating changes in biological activities and active ingredients upon processing were carried out for several herbs (Doui et al., 2010). Therefore, further investigation is necessary the ingredient analysis and identification of active compound to explain the different effect between a crude liquid form of DJ and a dried extract form of DJ.

Effect of DJ on T cell populations of spleen

The exposure on forced swimming test produces alterations in both cellular and non-cellular immunity (Connor et al., 1997). Single-positive T cells, which is expressing either CD4 or CD8, are mature T cells; Most $CD4^+CD8^-$ cells are Th cells responding to exogenous antigen in association with major histocompatiblity complex class II molecules, whereas, $CD4^-CD8^+$ cells respond to endogenous antigen in association with MHC class ² molecules and generally function as Tc cells (Luhtala et al., 1997). In this study, we performed the flow cytometry analysis to assess the T cell populations in spleen after force swimming test. The weights of spleen in the respective DJ-administered groups were similar to that of the control group (Table 1). No differences in the distribution of $CD4^+$ and $CD8^+$ cells in splenic cells were observed among the DJ-administered groups and saline-administered control group (Fig. 2). Therefore, this result suggested that DJ did not affect the population of T cell subsets in spleen isolated from mice.

Effect of DJ on cell viability and production of IFN-γ, IL-2, and IL-4 on splenocytes

Cytokines released from innate immune cells play important roles in the regulation of the *immune response*. Particularly cytokines released by Th cells at the onset of an immune response play decisive roles for pathological or physiological consequences. IFN-γ produced by Th1 cells is considered to be the principal effector cytokine of cell-mediated immunity, and many studies have indicated that it also plays an important role in controlling T cell homeostasis and apoptosis (Bernabei et al., 2003). Th1 cytokine, IL-2 is a T cell growth factor and it can augment natural killer cell cytolytic activity (Kim et al., 2006). In addition, IL-2 promotes immunoglobulin production by B cells and regulates the proliferation and apoptosis of activated T cells (Lenardo, 1991). IL-4 is required for the development and function of Th2 cells and plays an important role in allergy and immunoglobulin class switching (Holgate and Polosa, 2008). Finally, we investigated the effect of DJ on cytokine production from splenocytes. Cytotoxicity in the DJ-treated cells at the absence or presence ConA did not have the significant difference compared with the media control (Fig. 3A). To examine the effect of DJ on cytokine production

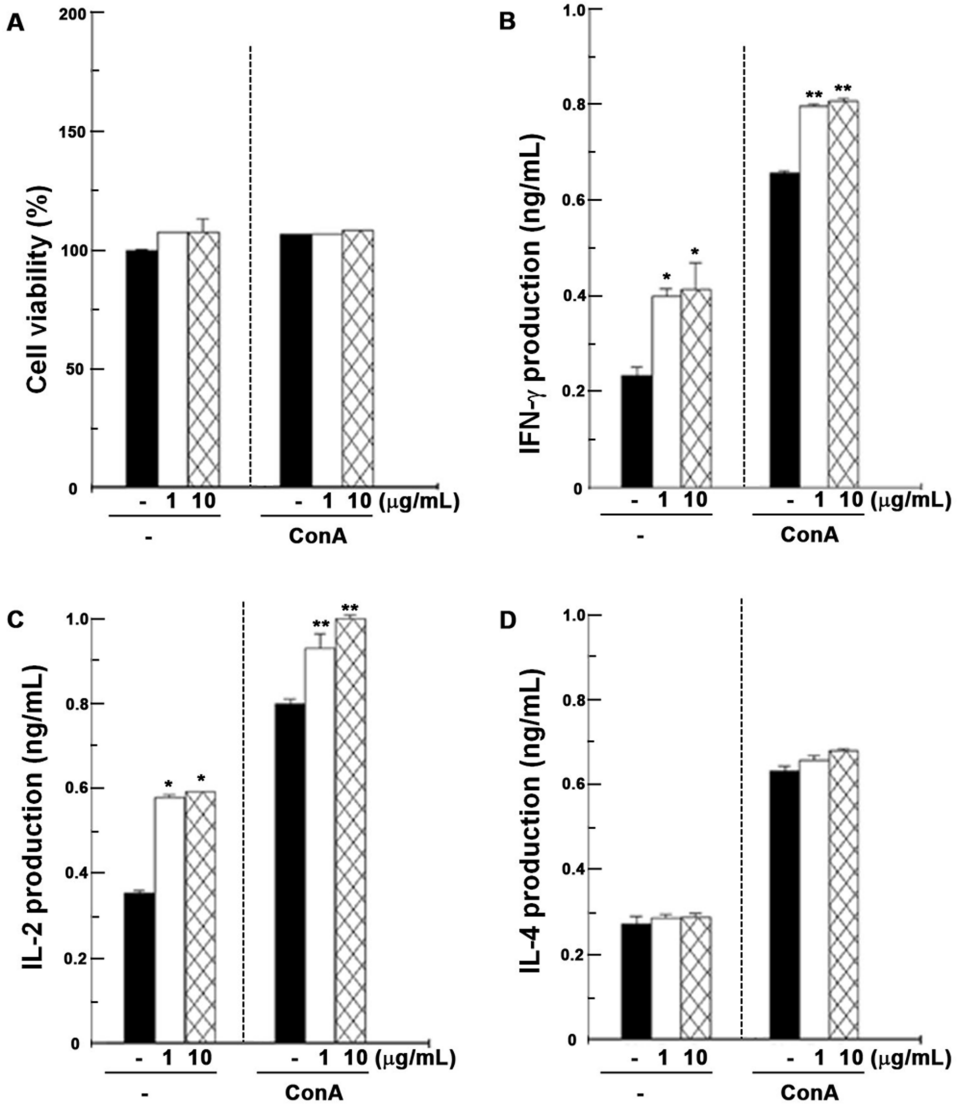

Fig. 3. Effect of DJ on cell viability and production of IFN-γ, IL-2, and IL-4 on splenocytes. Cells were stimulated with DJ as a final concentration of 1 mg/mL and 10 mg/mL for 24 h in the absence or presence of ConA (2 mg/mL). (A) Cell viability was determined with MTT assay. (B-D) Levels of cytokine were analyzed with ELISA. Values are means±SD. *p<0.05 *vs*. media control, **p<0.01 *vs.* ConA-treated group.

from splenocytes, DJ were treated in mouse splenocytes on the absence or presence of Con A (2 μg/mL) for 24 h. The production of IFN-γ, IL-2, and IL-4 was measured in the cell culture supernatants by ELISA (Fig. 3B-D). The levels of IFN-γ in DJ-treated cells showed a significant increase compared to that in the media controls (p<0.05, Fig. 3B). IL-2 levels were also increased by concentrations of 1 and 10 μg/mL DJ (p<0.05, Fig. 3C). DJ did not affect the IL-4 production in the absence or presence of ConA (p<0.05, Fig. 3D). In the figure 2, DJ had no effect on the population of T cell. On the other hand, DJ increased the levels of Th1 cytokines. Therefore, these findings suggest that DJ affects the immune function by increasing production of IFN-γ and IL-2 through activation of Th1 cells.

Conclusion

In this study, DJ reduced the immobility time and serum levels of LDH, CPK, and AST in the forced swimming test and increased the production of IFN-γ and IL-2 in splenocytes. Therefore, it suggests that the beneficial effects of DJ may have an immune-enhancing effect via activation of immune cells. However, further studies are required to elucidate the mechanism related to the change of cytokines and T cell function in forced swimming animal model.

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