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Immune-enhancement effect of JaSaengHwan

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SUMMARY

We investigated the immune enhancement effects of JaSaengHwan (JSH). The forced swimming test (FST) has been used as a screening model for new immune enhancement agents. We found that JSH (0.1 mg/ml) significantly reduced the immobility time in the FST compared to the control. Also, we investigated the effect of JSH on the proliferation of T cell and production of cytokines in human T-cell line, MOLT-4 cells and mouse peritoneal macrophages. JSH (1 mg/ml) significantly increased the cell proliferation by 46.78±6.41% (P<0.05) and also significantly increased the interleukin (IL)-2, IL-4 and interferon (IFN)-γ production compared with media control (about 2-fold for IL-2, 3-fold for IL-4 and 1.5-fold for IFN-γ, P<0.05) at 24 h. In addition, JSH increased the production of IL-12 on the mouse peritoneal macrophages (by 3.6-fold for IL-12, P<0.05). In conclusion, these data indicate that JSH may have an immune-enhancement effect.

Key Words: Immune enhancement; JaSaengHwan; Forced swimming test; Proliferation; Cytokines

INTRODUCTION

JaSaengHwan (JSH) is composed of 50% bamboo salt and traditional Oriental medicine-Allium sativum L., Ulmus pumila L., Atractylodes macrocephala K_{OIDZ}, Portulaca oleracea. Bamboo salt is a specially processed salt according to the traditional recipe using normal salt and bamboo in Korea. It is known to have various therapeutic effects on diseases such as inflammations, viral disease, diabetes, circulation organ disorder and cancer etc (Kim *et al*., 1993; Min *et al*., 1995; Yang *et al*., 1999; Huh *et al*., 2001). Although herbal medicines have long been used effectively in treating many diseases, the pharmacological mechanisms of most herbs used have not yet been defined.

The forced swimming test (FST) is commonly used to evaluate anti-depressants, and many anti-depressants show the anti-immobility effects (Prosolt *et al*., 1977; Borsini and Meli, 1988). An immobile posture observed in this test indicates 'behavioral despair' and can be an animal model of depression (Porsolt *et al*., 1977, 1978). In the present study, the immune-enhancement effect of JSH on immobility time in the FST was evaluated. Because JSH decrease the immobility time in this test without stimulating motor activity, it was postulated that JSH has a potential for inducing immune-enhancement.

Immunoregulatory cytokines play an important role in determining the nature and strength of an immune response (Paul *et al*., 1994; Abbas *et al*., 1996). Cytokines have historically been classified as Th1 type such as interleukin (IL)-2, interferon (IFN)-γ or Th2 type IL-4, IL-5, IL-6 based on studies originally involving cloned murine $CD4^+$ T cell subsets (Mosmann *et al*., 1986; Mosmann and Coffman, 1989). Th1 type cytokines promote cell-mediated immunity and IFN-γ induces B lymphocytes to switch from immoglobulin (Ig)M to IgG2a and IgG3 (opsonizing and complement-fixing subclasses). Th2 cytokines primarily facilitate the development of humoral immune responses. As described above, various cytokines such as IFN-γ, IL-2, IL-4, IL-12

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are related to immune reaction, direct or indirect.

The macrophage is involved in many different processes such as tissue remodeling during embryogenesis, wound repair, removal of damaged or senescent cells subsequent to injury or infection, haemopoiesis and homeostasis. Another function of macrophages is to provide a defense line against microbial invasion and to recognized and kill tumor cells (Adams *et al*., 1992). On the other hand, they play an indirect role in these anti-microbial or anti-tumor activities by secretion of cytokines (e.g. IL-12) or by antigen processing and presentation, thereby regulating the immune system (Klimp *et al*., 2002). IL-12 has been shown to play a central role in the innate and acquired immune responses. Its activities include enhancement of natural killer (NK) and cytotoxic T lymphocyte (CTL) activity and promotion of CD4 Th1 cell development. It has also been shown to provide potent activity as a vaccine adjuvant in generating antibody and T cell responses (Lee *et al*., 2000).

In the present study, the anti-immobility effects of JSH preformed the forced swimming test are to be investigated. To investigate the effect of JSH on the production of cytokines, the production of IL-2, IL-4, IFN-γ and IL-12 on the JSH treated MOLT-4 cells and mouse peritoneal macrophages was analyzed.

MATERIALS AND METHODS

Reagents

Avidin-peroxidase and 2'-AZINO-bis (3-ethylbenzi-thiazoline-6-sulfonic acid) were purchased from Sigma (St. Louis, MO, USA). RPMI 1640, ampicillin, streptomycin and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Anti-human IL-2, 4, IFN-g biotinylated anti-human IL-2, 4, IFN-γ and recombinant (r) human IL-2, 4, IFN-γ were purchased from R & D Systems (Minneapolis, MN, USA). Anti-human IL-12, biotinylated anti-human IL-12 and rhuman IL-12 were purchased from Pharmingen, USA.

Preparation of JSH

JSH was composed of 50% Bamboo salt, Allium sativum L., Ulmus pumila L., Atractylodes macrocephala K_{OIDZ}, Portulaca oleracea. Powdered JSH was melted in PBS, filtered through 0.45 μm filter, and kept 4°C.

Animals

The original stock of male ICR mice (10-12 g) were purchased from the Daehan Biolink Co., (Daejeon, Korea), and were housed at a room temperature of 23±1°C with a 12/12 hrs light-dark cycle (lights on from 6:00am to 6:00 pm). Food and water were available adlibitum.

MOLT-4 Cell culture

T cell line MOLT-4 cells were grown in RPMI 1640 medium (Gibco BRL, USA) supplemented with 10% fetal bovine serum (JRH BIOSCIENCE, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in the presence of 5% CO_2.

Peritoneal macrophage culture

TG-elicited macrophages were harvested 3 days after intraperitoneal injection of 2.5 ml TG to mice and isolated, as reported previously (Narumi *et al*., 1990). Using 8 ml of HBSS, which contained 10 U/ml heparin, performed peritoneal lavage. Then, the cells were distributed in DMEM, which was supplemented with 10% heat-inactivated fetal bovine serum (FBS), in either 4-well tissue culture plates (3×10^5 cells/well) incubated for 3 hrs at 37°C in an atmosphere of 5% CO_2, washed 3 times with HBSS to remove non-adherent cells, and equilibrated with DMEM that contained 10% FBS before treatment.

Forced swimming test (FST)

The forced swimming test was performed according to the methods described by Porsolt *et al*. (1977). Each mouse was placed in a 25-glass cylinder (10 cm diameter) containing 15 cm of water maintained at 23±1°C. Immobility was recorded during a 6-min swimming test.

Enzyme-linked immunosorbent assay (ELISA) of cytokines

Sandwich ELISA for IL-2, IL-4, IFN-γ, and IL-12 and was carried out in duplicate in 96-well ELISA plates (Nunc, Denmark) coated with each of 100 μl aliquots of anti-human IL-2, IL-4, IFN-γ and IL-12 monoclonal antibodies at 1.0 μg/ml in PBS at pH 7.4 and was incubated overnight at 4°C. The plates were washed in PBS containing 0.05% Teeen-20 (Sigma, St. Lousis, MO, USA) and blocked with PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3

for 1 h. After additional washes, sample or IL-2, IL-4, IFN-γ and IL-12 standards were added and incubated at 37°C for 2 h. After a 2 h incubation at 37°C, the wells were washed and then each of 0.2 μg/ml of biotinylated anti-human IL-2, IL-4, IFN-γ and IL-12 were added and again incubated at 37°C for 2 h. After washing the wells, avidin-peroxidase was added and plates were incubated for 20 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 made on each assay plate using recombinant IL-2, IL-4, IFN-γ and IL-12 in serial dilutions.

Statistical analysis

Effects of JSH on immobility were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's test. Other results were analyzed by the independent-*t* test. The experiments shown are a summary of the data from at least-three experiments. Values shown are means±standard error of the mean (SEM). Differences were considered statistically significant when *p* was less than 0.05.

RESULTS

Effect of JSH on immobility time in FST

In a first series of experiments, the forced swimming test had been used as a screening model. It was found that JSH (0.1, 1 g/kg) decreased the immobility time in the FST compared to the control (Fig. 1).

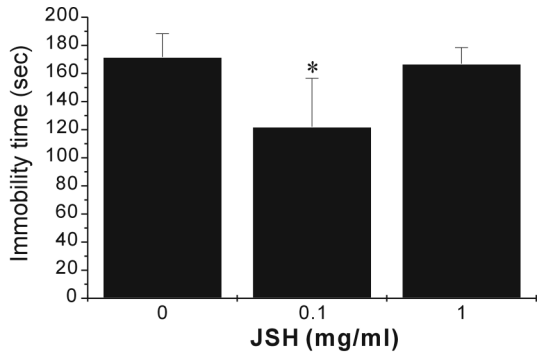

Fig. 1. Effect of JSH on immobility in forced swimming test. Immobility time recorded during 6 min in the FST in mouse given saline (control group, n=5) or JSH (n=5). Results are shown as mean±SEM. **P*<0.05; indicates significant difference from the saline group.

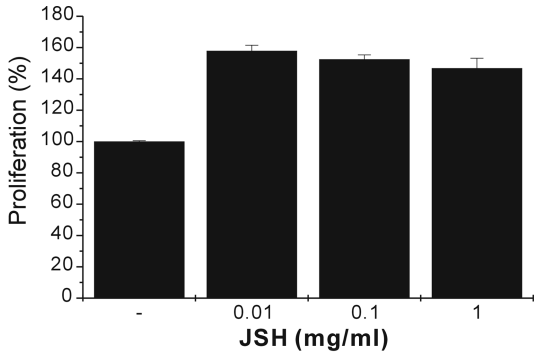

Fig. 2. Effect of JSH on the cell viability. MOLT-4 cells (3×10^5) were treated with various concentrations of JSH for 24 hrs. Cells were then collected and assessed for viability using MTT. Values are the mean±SEM of duplicate determinations from three separate experiments.

Apparent anti-immobility was observed following the administration JSH at 0.1 g/kg.

Effect of JSH on the proliferation of MOLT-4 cells

T-cells play a crucial role in immune functions as they act both as effectors and regulators (Riddell *et al.*, 2002). To assess the effect of JSH on the proliferation of T cell, the MTT assay was performed. As a result, JSH increased the proliferation of MOLT-4 by 46.78± 6.41% (at 1 mg/ml, $P<0.05$) (Fig. 2).

Effect of JSH on the production of IL-2, IL-4 and IFN-γ on MOLT-4

To assess the effects of JSH on the production of various cytokines, the MOLT-4 cells were treated with various concentrations of JSH for 24 h. The levels of IL-2, IL-4 and IFN-γ were analyzed by ELISA method. As shown in Fig. 3, JSH increased the IFN-γ production compared with media control (0.86±0.07 ng/ml) in a dose-dependent manner (1.23±0.13 ng/ml at 1 mg/ml). JSH also significantly increased the IL-2 and IL-4 production (0.77±0.13 ng/ml for IL-2 and 0.17±0.05 ng/ml for IL-4) compared with media control (0.42±0.05 ng/ml for IL-2 and 0.06±0.00 ng/ml for IL-4). The production was tended to increase at l mg/ml concentration of JSH, but IL-2 and IL-4 production enhancing effect of JSH became weaker at the low concentration (Figs. 4, 5).

Effect of JSH on the production of IL-12 on peritoneal macrophages

IL-12 is mainly produced by antigen-presenting

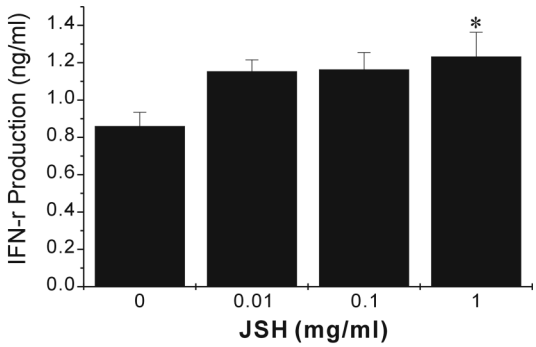

Fig. 3. Effect of JSH on IFN-γ production in the MOLT-4 cells. Culture supernatant was collected from none or JSH treated MOLT-4 cells, which were cultured for 24 hrs. Cytokines levels in culture supernatant was measured using ELISA. **P*<0.05; significantly different from the saline value.

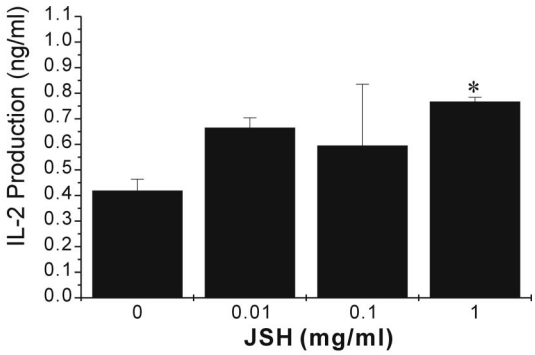

Fig. 4. Effect of JSH on IL-2 production in the MOLT-4 cells. Culture supernatant was collected from none or JSH treated MOLT-4 cells, which were cultured for 24 hrs. Cytokines levels in culture supernatant was measured using ELISA. **P*<0.05; significantly different from the saline value.

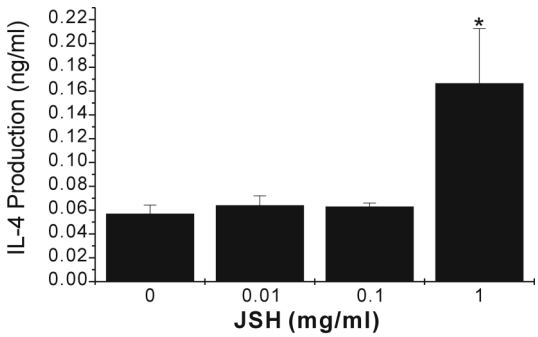

Fig. 5. Effect of JSH on IL-4 production in the MOLT-4 cells. Culture supernatant was collected from none or JSH treated MOLT-4 cells, which were cultured for 24 hrs. Cytokines levels in culture supernatant was measured using ELISA. **P*<0.05; significantly different from the saline value.

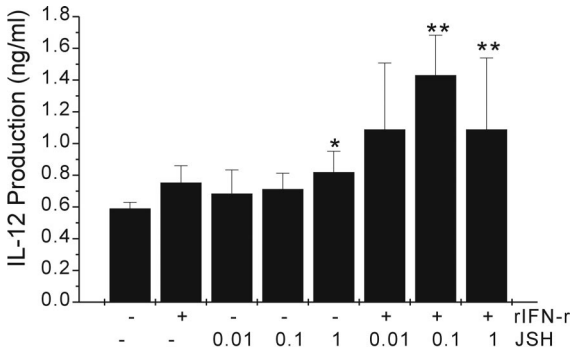

Fig. 6. Effect of JSH on IL-12 production in the mouse peritoneal macrophges. Peritoneal macrophages (3×10^5 cells / well) were stimulated with rIFN-γ, JSH or JSH +rIFN-γ. IL-12 levels in culture supernatant was measured using ELISA. 1, unstimulated cells; 2, rIFN-γ; 3, JSH (0.01 mg / ml); 4, JSH (0.1 mg / ml); 5, JSH (1 mg / ml); 6, JSH (0.01 mg / ml)+rIFN-γ; 7, JSH (0.1 mg / ml)+rIFN-γ; 8, JSH (1 mg / ml)+rIFN-γ. **P*<0.05 is different from unstimulated value. ***P*<0.05 is different from rIFN-γ treated value.

cells such as dendritic cells or macrophages, and plays an important role in eliciting the generation of Th1 cells (Furuya *et al*., 2001). Then probable ability of JSH to stimulate potential mediators such IL-12 in isolated mouse peritoneal macrophages was examined. Mouse peritoneal macrophages were cultured with rIFN-γ for 6 h and then stimulated with 0.01-1 mg / ml concentrations of JSH for 24 h. ELISA measured the amount of IL-12 secreted by the cells. As shown in Fig. 6, JSH alone did stimulate the production of IL-12 (unstimulated cells, 0.59±0.04 ng / ml for IL-12) in a dose-dependent manner. These productions were higher than rIFN-γ alone (rIFN-γ alone, 0.753±0.11 ng / ml for IL-12). The amount of IL-12 was significantly higher in the JSH plus rIFN-γ treated cells than rIFN-γ treated cells (about 1.5-fold for IL-12, $P<0.05$). Maximum effective concentration of JSH was 1 mg / ml for IL-2 production.

DISCUSSION

In the present study, it showed that JSH decreased the immobility time in the FST and increased the proliferation of T cells. JSH also strongly induced the production of IL-2, IL-4, IFN-γ, IL-12 from MOLT-4 and mouse peritoneal macrophages.

FST is considered to be a behavioral screening method for anti-depressants (Borsini and Meli, 1988). The results suggest that the decrease in the

immobility time caused by JSH in FST might be mediated through immune-enhancement.

The activation of tumor antigen-specific Th and Tc cells or non-specific macrophages and natural killer (NK) cells using immunotherapeutic approaches may lead to the subsequent destruction of tumor tissue (Dredge *et al*., 2002a). Previous reports have demonstrated that the induction of Th1-promoting cytokine, using specific adjuvants, can enhance anti-tumor immunity and can reduce or even prevent tumor growth (Dredge *et al*., 2002b). The immune response can be broadly categorized into a cellular or humoral mediated response. The production of IL-2, IFN-γ, TNF-α IL-12 lead to a Th1-type cellular response, while production of IL-4 and IL-6 lead to Th2-type humoral immunity (Romagnani, 1991; Parronchi *et al*., 1991; Zurawski and De Vries, 1994). Many cancer vaccines, particularly in combination with immune adjuvants, elicit strong cellular immune responses leading to the production of Th1 type cytokines such as IL-2, IFN-γ, TNF-α and IL-12 (Dalgeish, 2000). First of all, IL-2 cytokine (also known as T-cell growth factor) has multiple immunoregulatory functions and biological properties. IL-2, together with other factors and in conjunction with antigens, mitogens, or anti-Ig antibodies, controls B cell proliferation and differentiation into antibody-producing plasma cells (Jelinek and Lipsky, 1987). NK and lymphokine-activated killer cells, monocytes and macrophages all have the ability to respond to IL-2 with increased activity or proliferation (Kuziel and Greene, 1991; Minami *et al*., 1992). IFN-γ is also an important cytokine in the host defense against infection by viral and microbial pathogens (Samuel, 2001). IFN-γ induces a variety of physiologically significant responses that contribute to immunity. IL-12, which is primarily produced by activated macrophages and stimulates T cells and NK cells. It induces IFN-g and plays a role in promoting Th1 cell responses. Immunoregulation by IL-12 is of central importance in cell-mediated immunity against those pathogens and tumors that are controlled by cell mediated mechanisms (Mackensen *et al*., 1997). Previously it was reported that Th2 cytokines levels were higher than Th1 cytokines levels in various diseases including cerebral infarction (CI), allergy and asthma (Kim *et al*., 2000; Jeong *et al*., 2002). In this study, JSH strongly increased the production of Th1 cytokines. Therefore, these results suggest that JSH might have a beneficial effect in the treatment of various diseases (CI, asthma and cancer) through the immune-enhancement.

Proinflammatory cytokines involved in hemostatic and immunological imbalance lead to enlargement of various tissue damages. But IL-4 has been also called the prototypic immunoregulatory cytokine. Like many cytokines, it can affect a variety of target cells in multiple ways. IL-4 has an important role in regulating antibody production, hematopoiesis and inflammation, and the development of effector T-cell responses (Brown and Hural, 1997). In this study, the level of IL-4 in JSH treated cells was very higher compared with the control. Therefore, we can speculate that IL-4 increased by JSH may contribute to immune-enhancement response.

On the other hand, Th2 cytokine such as IL-4 and IL-10, inhibit the production of IL-2 and IFN-γ, while IFN-γ can interfere with the maturation of naive $CD4^+$ T cells into Th2 cells (Fiorentino *et al*., 1989; Heinzel *et al*., 1989; Fitch *et al*., 1993). In Previous report, Allergina increase the Th1 and Th2 cytokines production. In this study, JSH increased IL-4 and IFN-γ production. Even though the mechanism of this profile remains unknown, it can throught that JSH might have a kind of adaptogenic activity in immune system.

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