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Decrease of immobility behavior in forced-swimming test and immune system enhancing effect of traditional medicine Gamisipjundaebo-tang

Hye-Young Shin[a,1], Tae-Yong Shin[b,1], Sang-Wan Seo[a,c], Ho-Jeong Na[a], Yong-Taek Kwon[d], Bong-Keun Song[d], Eon-Jeong Lee[d], Yun-Kyung Kim[c], Moo-Chang Hong[a], Min-Kyu Shin[a], Seung-Heon Hong[c], Hyung-Min Kim[a,*]

[a]*College of Oriental Medicine, Kyung Hee University, 1 Hoegi-Dong, Dongdaemun-Gu, Seoul, 130-701, Republic of Korea*
[b]*College of Pharmacy, Woosuk University, Jeonju, Jeonbuk, 565-701, Republic of Korea*
[c]*College of Pharmacy, Wonkwang University, Iksan, Jeonbuk 570-749, Republic of Korea*
[d]*College of Oriental Medicine, Wonkwang University, Iksan, Jeonbuk, 570-749, Republic of Korea*

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Abstract

Gamisipjundaebo-tang (GSDBT) has been used for the purpose of development of physical strength. In the present study, we investigated the immune enhancing effect induced by GSDBT. We investigated the anti-immobility effect of GSDBT via a forced-swimming test and blood biochemical parameters related to fatigue, glucose, blood urea nitrogen, lactic dehydrogenase, creatine kinase, and total protein. GSDBT (0.1 and 1 g/kg) was orally administered to mice for 14 days. After 7 and 14 days, as assessed through a forced-swimming test, immobility time was decreased in the GSDBT-administrated group (0.1 and 1 g/kg) in comparison with the control group. In addition, after 8 days, the contents of glucose and lactate dehydrogenase in the blood serum were increased, and contents of blood urea nitrogen were decreased in the GSDBT-administrated group. After 15 days, the contents of glucose were increased, and the contents of lactate dehydrogenase and blood urea nitrogen were decreased in the GSDBT-administrated group. However, it had no effect on the elevation of creatine kinase and total protein level. We also investigated the effect of GSDBT on the production of cytokines in human T-cell line, MOLT-4 cells, and splenocytes. GSDBT significantly increased interferon (IFN)-γ and interleukin (IL)-2 levels compared with the media control but did not affect IL-4. GSDBT increased the protein expression of IFN-γ in MOLT-4 cells. These results suggest that GSDBT may be useful in immune function improvement and may also have antifatigue properties.

Keywords: Gamisipjundaebo-tang; Immune enhancement; Forced-swimming test; Cytokine

1. Introduction

Gamisipjundaebo-tang (GSDBT) is prepared from 15 medical herbs that tone the blood, increase energy, and strengthen overall health. This prescription has long been used traditionally against anemia, anorexia, extreme exhaustion, and fatigue (Choi et al., 2002). This original prescription reduces metastatic potential and enhances antibody production and cytotoxicity (Onishi et al., 1998; Hamada et al., 1988; Haranaka et al., 1988). Clinically, it has been demonstrated to improve the systemic general condition of cancer patients, and it also reduces the adverse effects of chemotherapy and radiation therapy (Yamada, 1994).

The forced-swimming test is perhaps one of the most commonly used animal models of behavioral despair and has been used extensively as a preclinical diagnostic tool

* Corresponding author. Tel.: +82 2 961 9448; fax: +82 2 968 1085.
E-mail address: hmkim@khu.ac.kr (H.-M. Kim).
[1] Both authors contributed equally to this work.

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for the assessment of novel antidepressants (Porsolt et al., 1978). Recently, the forced-swimming test has also been used as antifatigue and endurance tests (Dubovik and Bogomazov, 1987; Deyama et al., 2001; Kim et al., 2002; Ozturk et al., 2002). Glucose, blood urea nitrogen, lactate dehydrogenase, creatine kinase, and total protein are blood biochemical parameters related to fatigue. Energy for exercise is derived initially from the breakdown of glycogen and, later, from circulating glucose released by the liver and from nonesterified fatty acids (Dorchy, 2002). As is commonly known, glucose levels are decreased immediately after exercise. The blood urea nitrogen test is a routine test used primarily to evaluate renal function. Serum lactate dehydrogenase and creatine kinase are known to be accurate indicators of muscle damage (Burr et al., 1997; Coombes and McNaughton, 2000). Total protein is a rough measure of serum protein. Protein measurements can reflect nutritional state, kidney disease, liver disease, and many other conditions (Dorchy, 2002).

It has been reported that forced-swimming test exposure produces alterations in both cellular and noncellular immunity (Connor et al., 1998). Forced-swimming test exposure causes a reduction in the percentage of lymphocytes and an increase in the percentage of neutrophils in the peripheral blood, and there is a significant but transient suppression of both phytohemagglutinin- and concanavalin-A-induced lymphocyte proliferation following forced-swimming test exposure (Connor et al., 1997; Dubovik and Bogomazov, 1987). Forced-swimming test exposure produces a variety of time-dependent neurochemical, endocrine, and immune alterations in the rat (Connor et al., 1997).

Th1 lymphocytes produce interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor (TNF), which promote cell-mediated immunity. Th2 lymphocytes produce IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, and granulocyte macrophage-colony stimulating factor, which promote humoral antibody-mediated immune response (Carter and Dutton, 1996; Stephens et al., 2002). The induction of Th1 immune responses plays a critical role in protecting against various intracellular microorganisms and tumors, and also in reversing Th2 cell-facilitating diseases, such as allergic inflammation (Kang et al., 1999; Cohn and Ray, 2000). The Th1/Th2 classification has been useful in relating the overall patterns of cytokine production to clinical outcomes in a variety of pathological states (Abbas et al., 1996).

In the present study, we examined the anti-immobility effects of GSDBT during a forced-swimming test, and the blood serum contents of glucose, blood urea nitrogen, lactate dehydrogenase, creatine kinase, and total protein were subsequently assessed. To investigate the effect of GSDBT on the production of cytokines, we analyzed the production of IFN-γ, IL-2, and IL-4 on the GSDBT-treated MOLT-4 cells and splenocytes.

2. Materials and methods

2.1. Preparation of GSDBT

The plant materials were obtained from the Wonkwang Oriental Medicine Hospital (Jeonju, Jeonbuk) and authenticated by Professor E.J. Lee, College of Oriental Medicine, Wonkwang University. A voucher specimen (number 01-03-356) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University. An extract of GSDBT was prepared by decocting the dried prescription of herbs with boiling distilled water. The duration of decoction was about 3 h. The decoction was filtered, lyophilized, and kept at 4 °C. The yield of extraction was about 14% (w/w). The GSDBT water extract powder was dissolved in sterile saline (50 mg/ml). The ingredients of GSDBT include 5 g of Ginseng Radix Alba, Atractylodis Rhizom Alba, Hoelen, Glycyrrhizae Radix, Angelicae Gigantis Radix, Cnidii Rhizoma, Paeoniae Radix, and Rehmanniae Radix; 4 g of Astragali Radix, Cinnamomi Corte, Zingiberis Rhizoma, and Ziyphi Fructus; 3 g of Aurantii Nobilis Pericarpium, Linderae Radix, and Schizandra Chinensis.

2.2. Animals

The original stock of male ICR and BALB/c mice (4–6 weeks) was purchased from Daehan Biolink (Daejeon, Korea), and they were housed at a room temperature of 23±1 °C with a 12/12-h light–dark cycle (lights on from 6:00 a.m. to 6:00 p.m.). Food and water were available ad libitum. Mice were sacrificed in accordance with the National Institute of Health animal care and use guidelines.

2.3. Forced-swimming test

FST used was the same as described by Porsolt et al. (1977). Briefly, ICR mice were dropped individually into glass cylinders (height 25 cm, diameter 10 cm) containing 10 cm of water maintained at 23–25 °C, and left there for 6 min. The mouse was judged to be immobile when it floated in an upright position and made only small movements to keep its head above water. The duration of immobility was recorded during the last 4 min of the 6-min testing period. After the first measurement of immobility time, the mice were divided into control and GSDBT groups (0.1 and 1 g/kg) to match the swimming time in each group. Saline/GSDBT was given 24 h before the forced-swimming test was performed. Mice received their first saline/GSDBT oral administration (0.1 and 1 g/kg) for 14 days after the first forced-swimming test. In the second, third, and fourth forced-swimming tests, mice were forced to swim for a duration of 6 min, and immobility times were recorded by observers that were blind to the drug treatment. All experiments were carried out between 10:00 a.m. and 3:00 p.m. in testing rooms adjacent to the animal rooms.

2.4. Preparation and ingredient analysis of blood serum

After the forced-swimming test, the mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (4 mg/kg). After anesthetization, blood was withdrawn from the hearts of the forced-swimming-treated mice into syringes. Serum was then prepared by centrifugation at 3000 rpm at 4 °C for 10 min. Contents of glucose, blood urea nitrogen, lactate dehydrogenase, creatine kinase, and total protein were determined by an autoanalyzer (Hitachi 747, Hitachi, Japan).

2.5. MOLT-4 cell cultures

T cell line MOLT-4 cells were grown in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (JRH BIOSCIENCE, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL) at 37 °C in the presence of 5% CO_2. Cells were stimulated with GSDBT (0.01–1 mg/ml) for 24 h at 37 °C in 5% CO_2. Supernatants were harvested after 24 h for quantification of cytokine levels.

2.6. Splenocyte cultures

The spleen (BALB/c mice) was removed aseptically and teased into a single cell suspension in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 50 μM 2-ME (Sigma, St. Louis, MO, USA). 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 GSDBT (0.01–1 mg/ml) for 24 h at 37 °C in 5% CO_2. Supernatants were harvested after 24 h for quantification of cytokine levels.

2.7. Cytokine assay

IFN-γ, IL-2, and IL-4 (R & D Systems, Minneapolis, MN, USA) levels were assayed using a sandwich enzyme-linked immunosorbent assay (ELISA) following the instructions of the manufacturer. Briefly, 96-well ELISA plates (Nunc, Denmark) were coated overnight at 4 °C with antihuman (or mouse) IFN-γ, IL-2, and IL-4 monoclonal antibodies at 1.0 μg/ml in PBS at pH 7.4. The plates were washed in PBS containing 0.05% Teeen-20 (Sigma) and blocked with PBS containing 1% BSA, 5% sucrose, and 0.05% NaN_3 for 1 h. After additional washes, sample or IFN-γ, IL-2, and IL-4 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 antihuman (or mouse) IFN-γ, IL-2, and IL-4 was added and again incubated at 37 °C for 2 h. After washing the wells, avidin–peroxidase (Sigma) was added, and the plates were incubated for 20 min at 37 °C. Wells were again washed, and ABTS (Sigma) 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.

2.8. Western blot analysis

Cell extracts were prepared by a detergent lysis procedure. Cells (5×10^6 cells) were scraped, washed once with PBS, and resuspended in lysis buffer. Samples were vortexed for lysis for a few seconds every 15 min at 4 °C for 1 h and centrifuged at $15\,000\times g$ for 5 min at 4 °C. Samples were heated at 95 °C for 5 min and briefly cooled on ice. Following centrifugation at $15\,000\times g$ for 5 min, 50-μl aliquots were resolved by 12% SDS-PAGE. Resolved proteins were transferred overnight to a nitrocellulose membrane in 25 mM Tris, pH 8.5, 0.2 mM glycerin, and 20% methanol at 25 V. Blots were blocked for at least 2 h with 1×TBST containing 10% nonfat dry milk. Protein levels were analyzed essentially according to the manufacturer's instructions.

2.9. Statistical analysis

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

3. Results

3.1. Effect of GSDBT on immobility in the forced-swimming test

We investigated the anti-immobility effect of GSDBT in the forced-swimming test. GSDBT (0.1 and 1 g/kg) was orally administered to mice for 14 days. Measurement of immobility time was performed the next day (on the 8th and 15th days) after saline or GSDBT administration (on the 7th day, n=15; 14th day, n=8). The test results revealed that after 7 days, the immobility time was significantly decreased in the GSDBT-administrated group (0.1 and 1 g/kg) in a comparison with the saline-administrated group (Fig. 1). After 14 days, the immobility time was significantly decreased in the GSDBT-administrated group (0.1 g/kg). Apparent anti-immobility effects were observed following the administration of GSDBT at 0.1 g/kg (Fig. 1).

3.2. Effect of GSDBT on blood biochemical parameters

To clarify GSDBT's mechanisms, we assessed the levels of several blood biochemical parameters in mice after

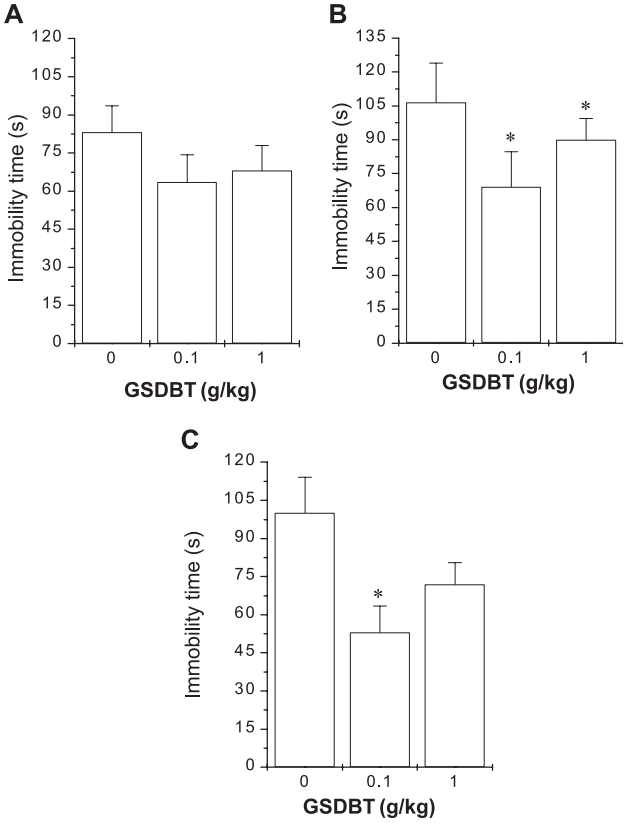

Fig. 1. Effect of GSDBT on forced-swimming-test-induced immobility in mice. One day after the first measurement of immobility, the administration of saline or GSDBT (0.1 and 1 g/kg) was started. Immobility time was measured after 1- (mice n=15, A), 7- (mice n=15, B), and 14-day (mice n=8, C) administration. Values are the mean±S.E.M. *P<0.05 indicates significant difference from the saline group.

forced-swimming tests. Blood was withdrawn from the hearts of forced-swimming-treated mice into syringes, and blood serum was prepared by centrifugation. The contents of glucose, blood urea nitrogen, lactate dehydrogenase, creatine kinase, and total protein were determined by an autoanalyzer. When GSDBT (1 g/kg) was administered orally for 7 or 14 days, blood urea nitrogen significantly decreased, and glucose level showed a tendency to increase; however, this increase was not significant. After 7-day GSDBT (1 g/kg) treatment, lactate dehydrogenase levels showed a significant increase, and after 14 days, lactate dehydrogenase levels were decreased in the GSDBT-treated group; however, this decrease was not significant. Low-dose (0.1 g/kg) GSDBT treatment was not found to be effective on each analyzed blood biochemical parameter (Tables 1 and 2).

3.3. Effect of GSDBT on the production of IFN-γ, IL-2, and IL-4 in the MOLT-4 cells

To assess the effects of GSDBT on the production of cytokines, MOLT-4 cells were treated with various concentrations of GSDBT for 24 h. The levels of IFN-γ, IL-2, and IL-4 were analyzed by ELISA method. As shown in Fig. 2A, GSDBT (0.01 and 1 mg/ml) significantly increased the IFN-γ level compared with the media control (about 2.1-fold for 0.01 mg/ml, 3.1-fold for 1 mg/ml; P<0.05). Fig. 2B shows that IL-2 level was significantly increased by GSDBT compared with the media control (about 2.8-fold for 1 mg/ml; P<0.05). However, GSDBT did not affect the production of IL-4 (Fig. 2C).

Table 1
Effect of GSDBT on blood biochemical parameters in mice after 7 days[a]

	Saline	GSDBT (0.1 g/kg/day)	GSDBT (1 g/kg/day)
Glucose (mg/dl)	246±12.1	245.5±4.5	303±15.3
Lactic dehydrogenase (U/l)	610.3±159.0	809.0±151	1540±290*
Blood urea nitrogen (mg/dl)	32.3±1.0	20.6±2.1	20.1±1.8*
Creatine kinase (mg/dl)	0.7±0.0	0.5±0.0	0.5±0.0
Total protein (g/dl)	5.3±0.3	4.8±0.2	5.1±0.2

[a] GSDBT (0.1, 1 g/kg for 7 days) was administered orally to mice; we have measured the levels of glucose, lactic dehydrogenase, blood urea nitrogen, creatine kinase and total protein in the serum after 7 days. Each level was determined by the autoanalyzer. Data represent mean±S.E.M (n=7, respectively).
* P<0.05 versus saline-administrated group.

3.4. Effect of GSDBT on the production of IFN-γ, IL-2, and IL-4 in the splenocytes

To examine the production of cytokines in splenocytes, GSDBT was treated with various concentrations for 24 h. The levels of IFN-γ, IL-2 and IL-4 were analyzed by the ELISA method. As shown in Fig. 3A and B, GSDBT (0.01–1 mg/ml) significantly increased the IFN-γ and IL-2 levels compared with the media control (about 5.8-fold at 0.01 mg/ml, 5.7-fold at 0.1 mg/ml, 6.8-fold at 1 mg/ml for IFN-ã level; about 4.7-fold at 0.01 mg/ml, 5.3-fold at 0.1 mg/ml, 5.8-fold at 1 mg/ml for IL-2; P<0.05). However, GSDBT did not affect the production of IL-4 compared with the media control (Fig. 3C).

Table 2
Effect of GSDBT on blood biochemical parameters in mice[a] after 14 days

	Saline	GSDBT (0.1 g/kg/day)	GSDBT (1 g/kg/day)
Glucose (mg/dl)	322.3±24.9	332±2.9	335.4±10.8
Lactic dehydrogenase (U/l)	973.2±133.2	705±128.8	530.7±129.5
Blood urea nitrogen (mg/dl)	21.5±0.8	19.4±0.8	18.6±0.8*
Creatine kinase (mg/dl)	0.7±0.0	0.6±0.0	0.6±0.0
Total protein (g/dl)	4.6±0.1	4.7±0.0	4.8±0.0

[a] GSDBT (0.1, 1 g/kg for 14 days) was administered orally to mice. We have measured the levels of glucose, lactic dehydrogenase, blood urea nitrogen, creatine kinase and total protein in the serum after 14 days. Each level was determined by the autoanalyzer. Data represent mean±S.E.M. (n=8, respectively).
* P<0.05 versus saline-administrated group.

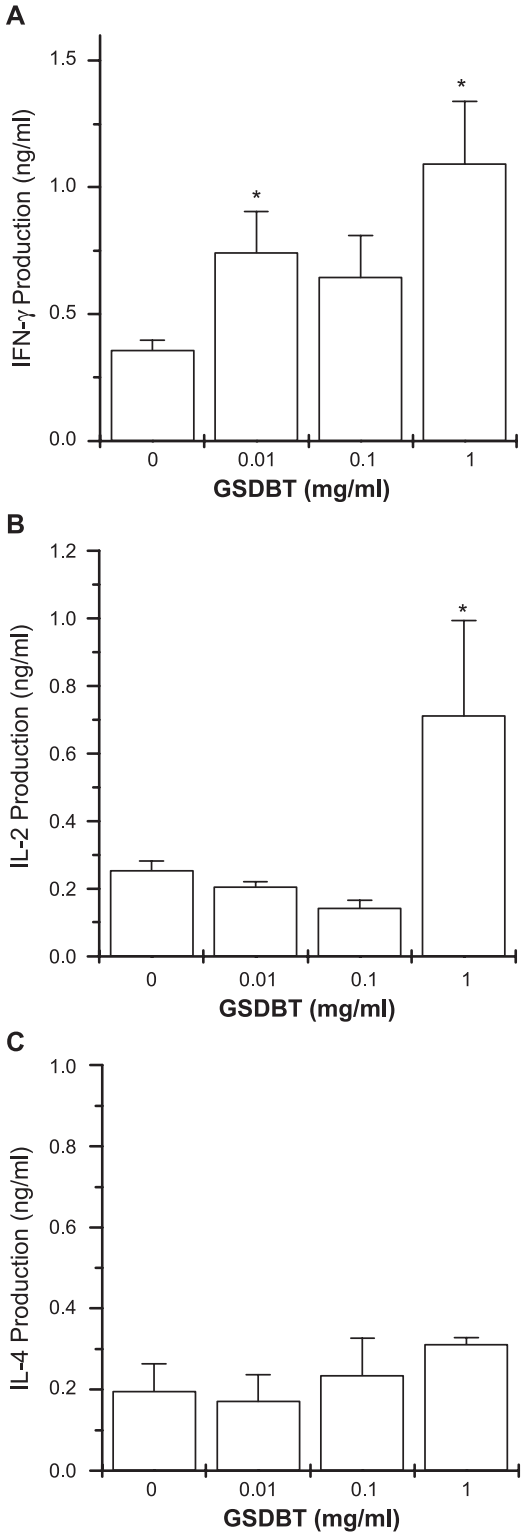

Fig. 2. Effect of GSDBT on IFN-γ (A), IL-2 (B), and IL-4 (C) production in the MOLT-4 cells. Culture supernatant was collected from none or GSDBT-treated MOLT-4 cells, which were cultured for 24 h. Cytokines levels in the culture supernatant was measured using ELISA. Data represent mean± S.E.M. of five independent experiments. *P<0.05, significantly different from the saline value.

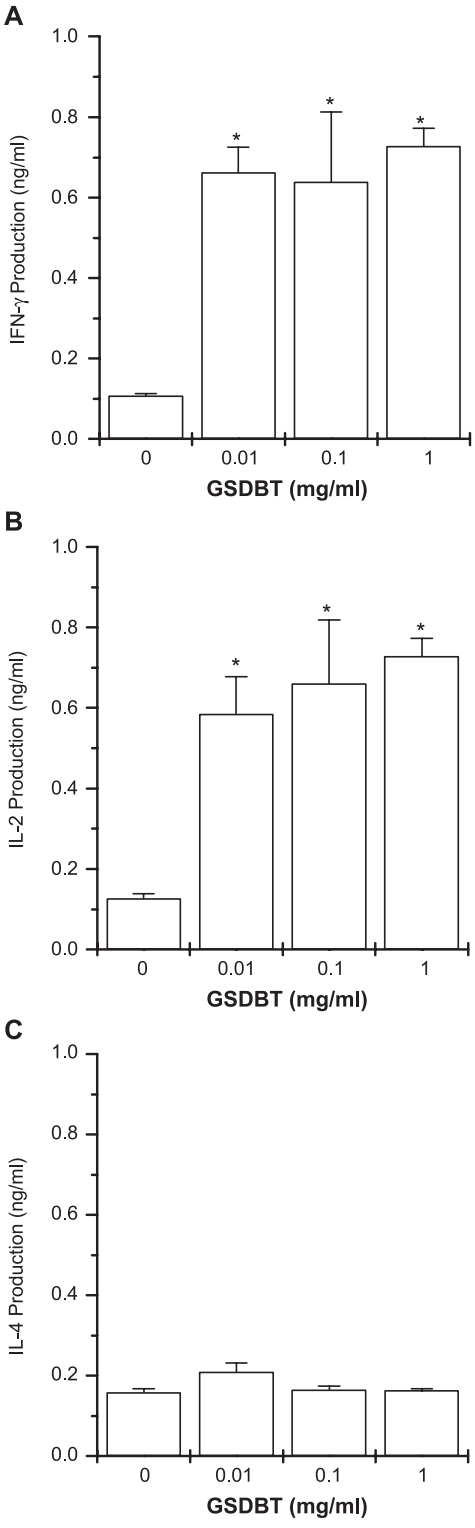

Fig. 3. Effect of GSDBT on IFN- γ(A), IL-2 (B), and IL-4 (C) production in the splenocytes. Culture supernatant was collected from none or GSDBT-treated splenocytes, which were cultured for 24 h. Cytokines levels in the culture supernatant was measured using ELISA. Data represent mean± S.E.M. of five independent experiments. *P<0.05, significantly different from the saline value.

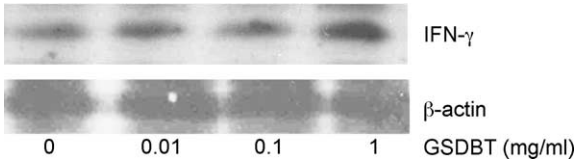

Fig. 4. Effect of GSDBT on IFN-γ expression in the MOLT-4 cells. The protein extracts were prepared, and samples were analyzed for IFN-γ expression by western blotting, as described in the Materials and methods section.

3.5. Effect of GSDBT on IFN-γ protein expression in the MOLT-4 cells

IFN-γ protein levels of intracellular of MOLT-4 cells were determined by Western blot analysis (Fig. 4). Treatment of cells with GSDBT (1 mg/ml) increased IFN-γ expression.

4. Discussion

The results of the current study show that the duration of immobility was shortened by the administration of GSDBT. These phenomena suggest that the decreased duration of immobility in mice may be caused by a change of certain metabolites in the system. After 7 days, lactate dehydrogenase increased, and blood urea nitrogen level in the blood serum decreased significantly (Table 1). After 14 days, only the blood urea nitrogen level decreased significantly (Table 2). The lactate dehydrogenase is known to be an accurate indicator of muscle damage and catalyzes the interconversion of pyruvate and lactate. Lactate serves as an energy source in highly oxidative tissue (Dorchy, 2002; Burr et al., 1997). Lactate dehydrogenase levels can increase in muscle after exercise in rodents and humans (Lawler et al., 1993). Blood urea nitrogen and lactate dehydrogenase levels of rugby players during a summer training camp significantly increased (Mashiko et al., 2004). Interestingly, the lactate dehydrogenase level tended to decrease by the administration of GSDBT for 14 days. On the other hand, the glucose level decreased immediately after exercise. Our results showed that glucose level tended to increase by the administration of GSDBT (1 g/kg), but the statistical difference was weak (8 days, P=0.09; 15 days, P=0.59). These data suggest that GSDBT may act as an energy source. In general, swimming exercise is known to induce blood biochemical changes (Kumahara et al., 1989). Although the statistical difference was weak, tendencies among some changes were found in biochemical parameters after 7- and 14-day application with GSDBT. This change appears to be related to the decrease of immobility time. As such, our results indicate that fatigue metabolisms of mice were influenced by GSDBT administration. However, further studies to clarify the detailed mechanisms involved in the antifatigue-like properties of GSDBT are necessary to support the present findings.

Many psychotropic drugs have been screened in the forced-swimming test (Porsolt et al., 1977; Borsini et al., 1991). Although many antidepressants attenuate forced-swimming-test-induced immobility, little is known about the forced-swimming-test-induced immune alteration (Borsini et al., 1991). Some studies have reported reduced neutrophil phagocytosis, impaired natural killer cell cytotoxic responses, suppression of lymphocyte proliferation, and IL-2 production as a result of exposure to the forced-swimming test (Shu et al., 1993). Many similar alterations in immune function have been reported in depressed patients (Irwin et al., 1987; Kronfol and House, 1989).

Immunoregulatory cytokines play an important role in determining the nature and strength of an immune response (Abbas et al., 1996; Paul and Seder, 1994). Recent studies indicate that the ratio of these two Th cell types, Th1 and Th2, is closely correlated with the outcome of many diseases, and controlling the Th1/Th2 ratio has been demonstrated as a therapeutic strategy for various diseases (Singh et al., 1999; Spellberg and Edwards, 2001; Boothby et al., 2001). Many cancer vaccines, particularly in combination with immune adjuvants, elicit strong cellular immune responses, leading to the production of Th1-type cytokines, such as IFN-γ, IL-2, and TNF-α (Dalgleish, 2000). IFN-γ is also an important cytokine in host defense against infection by viral and microbial pathogens (Samuel, 2001). Previously, we reported that Th2 cytokine levels were higher than Th1 cytokine levels in various diseases, including cerebral infarction, allergies, and asthma (Kim et al., 2000; Jeong et al., 2002). In this study, we showed that GSDBT strongly increased the production of IFN-γ and IL-2 while it did not affect the production of IL-4 in MOLT-4 cells and splenocytes (Figs. 2 and 3). There is no dose dependency for the most part in the actions of GSDBT, except IL-2 production in splenocytes. Despite the absence of dose dependency, those changes are, however, noteworthy as evidence of GSDBT affecting the immune system. The effective concentration on the cytokine production is too high, which reduces the likelihood of its utilization in practical applications. We believe that the results of this study may provide us with a clue toward understanding the pharmacological function of the formula for future in vivo study.

In the present study, we showed that GSDBT decreased the immobility time in the forced-swimming test. GSDBT also strongly induced the production of IFN-γ and IL-2 from MOLT-4 cells and splenocytes. Our results suggest that the decrease in the immobility time caused by GSDBT administration in the forced-swimming test might be mediated through immune enhancement.

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