

Immunomodulatory and Energy-Enhancing Effects of Modified SOUL-tang

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ABSTRACT

Modified SOUL-tang (MST) has been known to improve recovery of energy, regulation of body heat, and improvement of qi circulation, but the scientific evidence of MST has not been established. Here, we aimed to determine the energy-regulating effect of MST on performance in the forced swimming test (FST) and on RAW264.7 macrophage activity. MST was administered orally once a day for 28 days. On the 28 days, the immobility time in the FST was significantly decreased in the MST-fed group in comparison with the control group. MST induced a significant increase in serum interferon- γ , interleukin-2, and tumor necrosis factor (TNF)- α levels compared with the control group. *In vitro* experiments demonstrated that MST significantly increased the expression of TNF- α , nitric oxide (NO), and inducible NO synthase in RAW264.7 macrophages. Furthermore, MST stimulation induced the phosphorylation of I κ B α , subsequently promoting the nuclear translocation of nuclear factor- κ B. These results indicate that MST enhances energy by boosting immune function, suggesting its potential as an energizer for individuals with energy deficiency.

Keywords MST, FST, interferon- γ , nitric oxide, nuclear factor- κ B

INTRODUCTION

Immunodeficiency is characterized by diminished the immune system and allows the infections by fungi, viruses, and bacteria.¹ Immunodeficiency is mostly acquired but some people are born with defects in their immune system. Humoral and cell-mediated immunity were reduced in disorders associated with immunodeficiency.² T cells play an important role in immune functions, and helper T cells (Th cells) have two distinct subsets, Th1 and Th2.³ Th1 cells produce Th1 cytokines such as interferon (IFN)- γ , interleukin (IL)-2, and tumor necrosis factor (TNF)- α , which increase cell-mediated immunity.⁴ Th2 cytokines released from Th2 cells promote humoral antibody-mediated immune response.⁵ T cell deficiency also causes acquired immune deficiency syndrome⁶. Reduced T cell activity leads to impaired mitochondrial function, resulting in decreased ATP production and ultimately causing energy deficiency.⁷

Macrophages act as the first line of defense against microbial

invasion and induce apoptosis of microorganisms or cancer cells through the release of nitric oxide (NO) and TNF- α .⁸ NO has multiple biological functions, and it was synthesized by inducible NO synthase (iNOS).⁹ The expressions of TNF- α and NOS were transcribed by nuclear factor- κ B (NF- κ B). Phosphorylation of I κ B by cytokines, drugs, viruses, and bacterial products rapidly results in I κ B degradation and translocation of NF- κ B to the nucleus.¹⁰

The modified SOUL-tang (MST), which is made by reducing the herbal ingredients in SOUL-tang, is known to aid in the recovery of energy, regulation of body heat, and improvement of qi circulation. Additionally, MST is known for its immune-enhancing effects, helping to support overall vitality and strengthen the body's natural defenses, and is used for patients with reduced energy levels. Therefore, maintaining a healthy immune system helps prevent fatigue and allows the body to sustain energy more efficiently. However, scientific evidence supporting MST has not yet been established.

Forced swimming test (FST) is a behavioral test for evaluation of novel antidepressants and immune-enhancing drugs.¹¹ The lower blood levels of free triiodothyronine and fT4 (free thyroxine) observed in the FST may be linked to reduced energy production due to their role in regulating metabolism and energy generation.¹² In the present study, we examined the energy-enhancing effect of MST in FST. Furthermore, we

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investigated the effects of MST on production of NO and TNF- α and activation of NF- κ B in RAW264.7 macrophages.

MATERIALS AND METHODS

Preparation of MST

The MST was prescribed by Dr. Joohong Park, and MST decoction was provided by SOUL Korean Medical Hospital (Seoul, Republic of Korea). MST is composed of *Platyclusus orientalis* L. (16 g), *Poria cocos* Wolf. (8 g), *Euphoria longan* (Lour.) Steud. (24 g), *Rehmannia glutinosa* (Gaertn.) DC. (8 g), *Cuscuta chinensis* Lam. (16 g), *Cornus officinalis* Siebold & Zucc. (8 g), and *Rubus coreanus* Miq. (6 g). The decoction was subsequently filtered and lyophilized, resulting in a yield of approximately 4.08%. MST (100 mg/ml) was dissolved in distilled water and filtered using a 0.22 μ m syringe filter.

Animals

We purchased male ICR mice (10-12 g, 3 weeks old) from the Dae-Han Experimental Animal Center (Daejeon, Korea). Experiments were initiated after 1 week to allow for adaption to the laboratory environment. Animals were housed five per cage in a laminar air-flow room maintained at a temperature of $22 \pm 1^\circ\text{C}$ at a relative humidity of $55 \pm 10\%$ under a 12:12 L/D cycle light on at 07:00 h throughout the study. Food and water were available ad libitum. All manipulations were carried out between 09:00 and 16:00 h, and no animal was used more than once. All protocols were approved by the institutional animal care and use committee of Kyung Hee University (KHUASP(SE)-10-032). The research was conducted in accordance with Canadian Council on Animal Care (CCAC) guidelines.

FST

After the first measurement of immobility times, the mice were randomly divided into control group, *Chlorella vulgaris* extract (CVE, 0.3 g/kg) group, and MST (0.01, 0.1, and 1 g/kg) group based on the recorded swimming times. The CVE was supplied by Daesang Corp. WellLife (Seoul, Korea). It was dissolved in D.W. and used as a positive control. MST (0.01, 0.1, and 1 g/kg), CVE (0.3 g/kg), or D.W. was orally administered to mice once a day for 4 weeks using an atraumatic feeding needle. The FST was performed at 0- and 28-days administration period. MST, CVE, and D.W. were administered 1 h prior to the FST. During the 6 min of the FST, the immobility time was analyzed as previously described by.¹³ The FST was recorded with a Canon Camcorder. The immobility times were measured with a stopwatch by a trained observer who was blind to the experimental treatments. There were five mice in each group.

Enzyme-linked immunosorbent assay (ELISA)

Cytokines in the serum and supernatant were measured by ELISA. ELISA was performed as described previously.¹⁴

Cell culture

RAW264.7 cells, a macrophage-like cell line, were grown in DMEM (Gibco BRL, Grand Island, NY) with 10% heat inactivated fetal bovine serum, 1% penicillin-streptomycin at 37°C in 5% CO_2 and 95% air. RAW264.7 cells (3×10^5) were treated with MST (0.01, 0.1, and 1 mg/ml) and lipopolysaccharide (10 $\mu\text{g/ml}$, LPS) for 24 h.

Reverse transcription-PCR analysis (RT-PCR)

Using an easy-BLUE™ RNA extraction kit (iNtRON Biotech, Sungnam, Korea), total RNA from RAW264.7 cell was isolated according to the manufacturer's specifications. We performed RT-PCR with the following primers for mouse TNF- α (3'-TAC AGG CTT GTC ACT CGA AT-3'; 5'-ATG AGC ACA GAA AGC ATG AT-3'); GAPDH (5'-GGC ATG GAC TGT GGT CAT GA-3'; 5'-TTC ACC ACC ATG GAG AAG GC-3'). The annealing temperature was 62°C for TNF- α and 60°C for GAPDH. Products were electrophoresed on 1.5% agarose gel.

Measurement of nitrite concentration

RAW264.7 cells were stimulated with various concentrations of MST (0.01, 0.1, and 1 mg/ml) or LPS for 48 h. NO production in cell cultures was measured by a Griess method, as previously described.¹⁵

Western blot analysis

RAW264.7 cells were stimulated with MST or LPS for 1 h. Samples were heated at 95°C for 5 min and briefly cooled on ice. After the centrifugation, 50 μg aliquots were resolved by 12% SDS-polyacrylamide gel electrophoresis. The resolved proteins were electro-transferred overnight to nitrocellulose membranes in 25 mM Tris, pH 8.5, 200 mM glycerol, and 20% methanol at 25 V. Blots were blocked for at least 2 h with $1 \times$ phosphate-buffered saline containing 0.05% Tween 20 containing 5% nonfat dry milk and then incubated with NF- κ B, histone, phosphorylated I κ B, iNOS, and tubulin antibodies (Santa Cruz, CA, USA) for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibodies, and proteins were visualized by enhanced chemiluminescence procedures, (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions.

Statistical analysis

Results were expressed as the means $\pm$ SEM. Statistical significance was compared among each treated group and controlled by an independent *t*-test and an ANOVA with a Tukey post hoc test using SPSS statistical software (SPSS Inc., Chicago, IL, USA). The results were significant with a value of $P < .05$.

RESULT

Effect of MST on immobility during FST

We investigated the effect of MST in the immobility time during the FST. MST (0.01, 0.1, and 1 g/kg) or CVE (0.3 g/kg) were orally administered to mice once a day for 28 days. Measurement of immobility time was performed 1 h after MST or CVE administration. The immobility time revealed a significant decrease in the MST-administered group (0.1 and 1 g/kg) in comparison with the control group (Fig. 1, $P < .05$). CVE significantly reduced the immobility time (Fig. 1, $P < .05$).

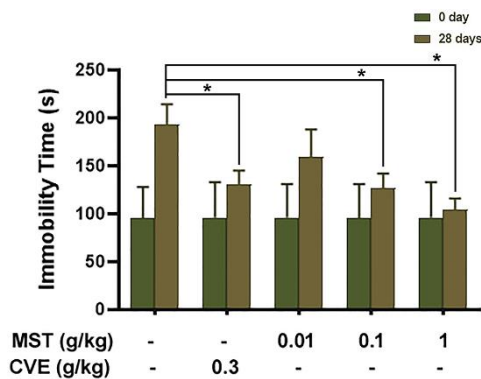


Fig. 1. Effect of MST on the FST-induced immobility in mice. Immobility time on FST. MST or CVE were administered 1 h before the test. Values are means $\pm$ SEM. * $P < .05$ vs. the D.W.-administered control group. MST. Modified SOUL-tang.

Effect of MST on levels of serum Th1 cytokines

Th1 cytokines secreted by Th1 cells increase the cell-mediated immune response⁴. Thus, we analyzed the levels of Th1 cytokines (IFN- γ , IL-2, and TNF- α) in the serum after FST. MST (1 g/kg) significantly increased the levels of serum IFN- γ , IL-2, and TNF- α in a dose-dependent manner (Fig. 2, $P < .05$). Furthermore, TNF- α levels were significantly increased at concentration of MST 0.1 g/kg (Fig. 2C, $P < .05$).

Effect of MST on production of TNF- α and NO in RAW264.7 cells

Macrophages directly control the immune system through their innate immune functions, and activated macrophages secrete TNF- α and NO⁸. To evaluate the effect of MST on the production of TNF- α , RAW264.7 cells were treated with various concentrations (0.01, 0.1, and 1 mg/ml) of MST for 24 h. As shown Fig. 3A, MST (0.01, 0.1, and 1 mg/ml) significantly increased the TNF- α production compared with the unstimulated cells ($P < .05$). The mRNA levels of TNF- α were increased by treatment with MST (Fig. 3B). To determine the effect of MST on the production of NO, RAW264.7 cells were treated with

various concentrations (0.01, 0.1, and 1 mg/ml) of MST for 48 h. MST significantly increased the NO production in a dose-dependent manner (Fig. 3C, $P < .05$). MST (1 mg/ml) or LPS (10 μ g/ml) also increased iNOS expression in the RAW264.7 cells (Fig. 3D).

Effect of MST on NF- κ B activation in RAW264.7 cells

NF- κ B is a transcription factor that regulates the expression of TNF- α and NOS and plays a central role in immunity¹⁵. Thus, we examined the effect of MST on NF- κ B activation in the RAW264.7 cells. Stimulation with MST induced the translocation of NF- κ B(p65) to the nucleus following the phosphorylation of I κ B α (Figs. 4 and 5).

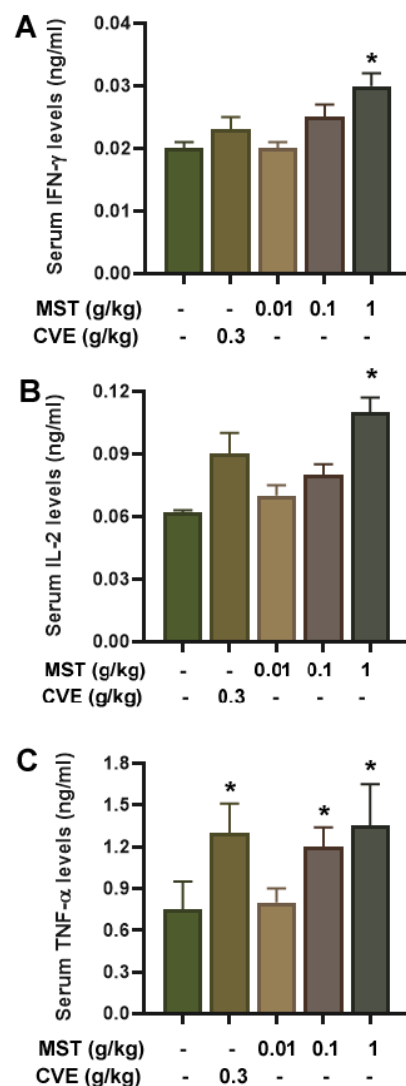


Fig. 2. Effect of MST on the levels of Th1 cytokine in the serum. (A-C) The levels of IFN- γ , IL-2, and TNF- α in the serum after FST were measured using an ELISA. Values are means $\pm$ SEM. * $P < .05$ vs. the D.W.-administered control group. MST. Modified SOUL-tang.

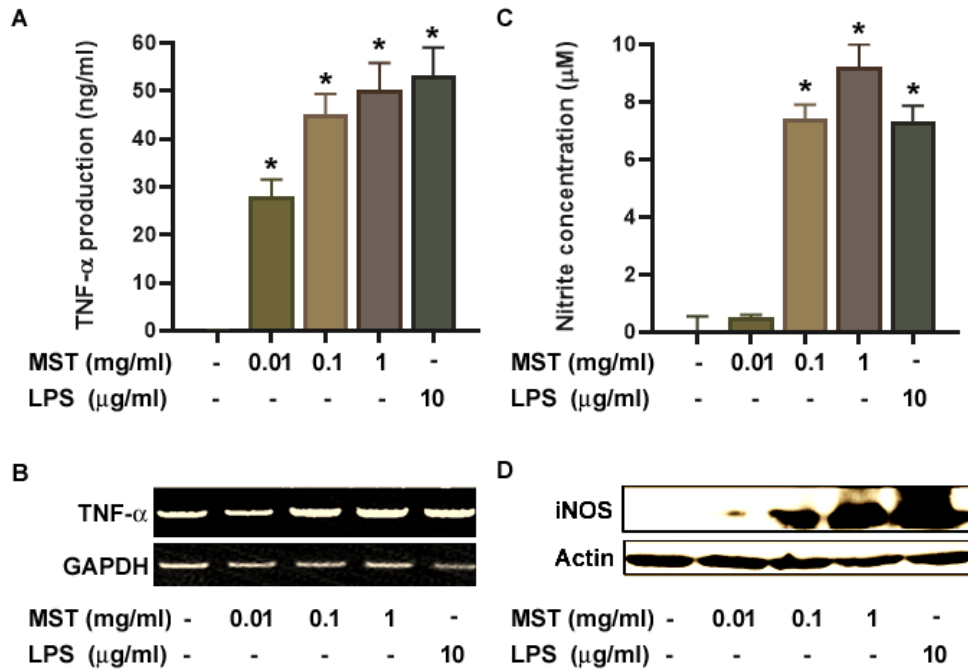


Fig. 3. Effect of MST on the levels of TNF- α and NO in the RAW264.7 cells. RAW264.7 cells were treated with various concentrations (0.01, 0.1, and 1 mg/ml) of MST. (A) The TNF- α production was analyzed using an ELISA and (B) TNF- α mRNA expression was analyzed using a RT-PCR. Results are representative of three independent experiments. (C) NO production was measured by the Griess method (nitrite). (D) Total proteins were determined for iNOS expression by Western blotting. Data are mean $\pm$ SEM values of three independent experiments performed in duplicate. * $P < .05$ vs. the unstimulated cells. MST, Modified SOUL-tang.

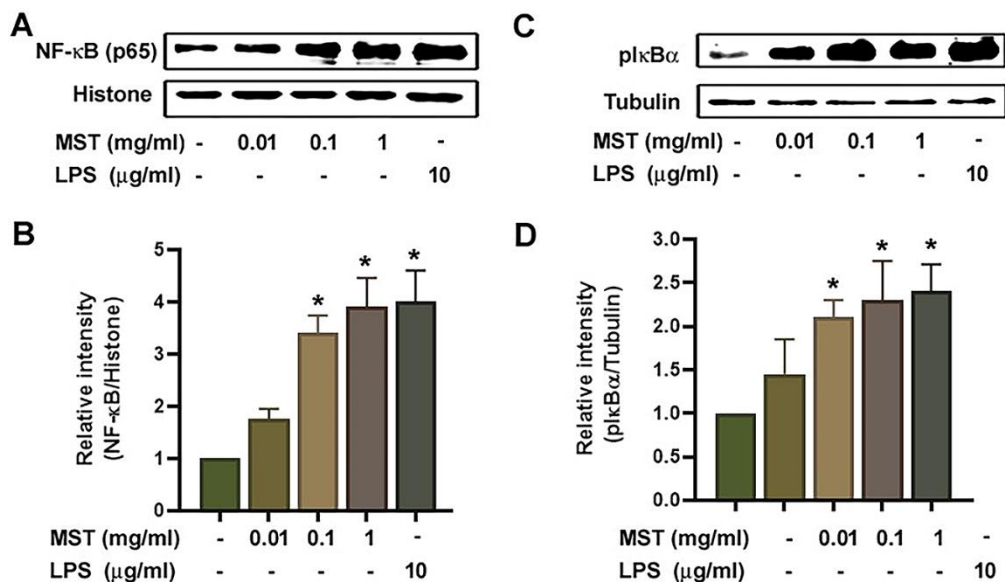


Fig. 4. Effect of MST on the activation of NF- κ B in the RAW264.7 cells. RAW264.7 cells were treated with MST (0.01, 0.1, and 1 mg/ml) or LPS (10 μ g/ml) for 1 h. (A) The nuclear extract was analyzed for NF- κ B by Western blotting. (B) The relative protein levels between NF- κ B and histone were quantified by densitometry. (C) Cytoplasmic extract was analyzed for pI κ B α by Western blotting. (D) The relative protein levels of pI κ B α and tubulin were quantified by densitometry. Data are mean $\pm$ SEM values of three independent experiments performed in duplicate. Results are representative of three independent experiments. * $P < .05$ vs. the unstimulated cells. MST, Modified SOUL-tang.

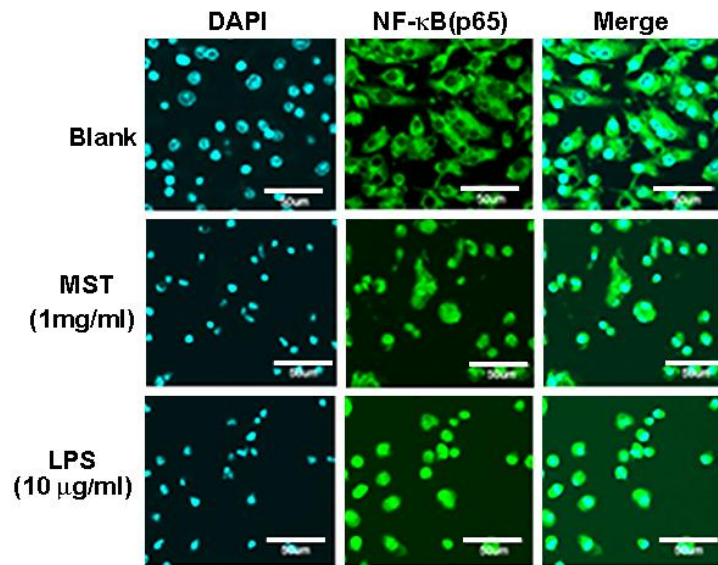


FIG. 5. Effect of MST on the NF- κ B translocation into nuclear in the RAW264.7 cells. RAW264.7 cells were treated with MST (1 mg/ml) and/or LPS (10 μ g/ml) for 1 h. NF- κ B was stained by a primary anti-p65 for 1 h and then incubated with secondary FITC-conjugated IgG for 30 min. Results are representative of three independent experiments. Blank, unstimulated cells (Original magnification $\times$ 400, scale bar = 50 μ m). MST. Modified SOUL-tang.

DISCUSSION

Many psychotropic drugs have been developed using the FST.¹¹ Some researchers have reported that exposure to FST attenuates lymphocyte proliferation and IL-2 production, damages natural killer cell cytotoxic responses, and reduces neutrophil phagocytosis.^{16,17} Panax ginseng has been used as a traditional Korean medicine for improving physical stamina, enhancing immunity, and enhancing energy. The immobility time in FST was reduced in Panax ginseng-administered mice.¹⁸ In this study, MST reduced the immobility time during the FST. Therefore, this result indicated that MST may have an energy-enhancing effect.

Th1 cells are key factor in the cellular immune response and play a central role in host defense systems for various pathogens.⁵ Th1 cytokines such as IFN- γ , IL-2, and TNF- α , play a vital role in regulation of immune response and activate lymphocytes, macrophages, and polymorphonuclear cells to destruct bacterial pathogen.⁴ IFN- γ is a definitive cytokine in the immune responses against infection by viruses, bacteria, parasites, or tumor cells.¹⁹ IL-2 induces differentiation and proliferation of B cell.²⁰ TNF- α attended the host defense against pathogens and triggered the immune responses via increasing cytokines production.²¹ In the present study, we showed that MST induced a significant increase in the levels of serum Th1 cytokine. These results suggest that MST might have a useful effect in the treatment of cancer and infection diseases via the energy-enhancing effect.

Macrophages are involved in homeostasis, wound repair, tissue remodeling during embryogenesis, and removal of damaged or senescent cells subsequent to injury or infection.⁸ NO expands blood vessels, improving blood flow, which allows

oxygen and nutrients to be delivered more effectively to the cells. This contributes to enhanced endurance during exercise and ultimately helps improve physical strength and energy.²² In this study, MST increased the production of NO and TNF- α and expression of iNOS and TNF- α . Furthermore, the phosphorylation of I κ B α and activation of NF- κ B were induced by MST in the RAW264.7 cells. Therefore, we speculate that NO and TNF- α increased by MST may contribute to energy-enhancement metabolism.

In conclusion, we showed that MST significantly reduced immobility times in a dose-dependent manner on the 28 days. MST significantly increased the serum IFN- γ , IL-2, and TNF- α levels. Additionally, MST significantly increased the production of TNF- α and NO and expression of TNF- α and iNOS via the activation of NF- κ B in the RAW264.7 cells. Therefore, we suggest that MST may have potential as an essential medicine for enhancement of energy metabolism.

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CONFLICT OF INTEREST

The authors declare no competing interests.

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