

## The effect of *Panax ginseng* on forced immobility time & immune function in mice

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**Background & objectives:** *Panax ginseng* has been used as a traditional medicine for many years mainly among Asian peoples for developing physical strength. We undertook this study to determine the immune-enhancement effect of *P. ginseng* using a forced swimming test (FST) and by measuring cytokine production in MOLT-4 cell culture and mouse peritoneal macrophages.

**Methods:** *P. ginseng* was orally administered to mice once a day for 7 days. The anti-immobility effect of *P. ginseng* on the FST and blood biochemical parameters related to fatigue, glucose (Glc); blood urea nitrogen (BUN); latic dehydrogenase (LDH); total protein (TP) and production of cytokines in human T cell line, MOLT-4 cells and mouse peritoneal macrophages were investigated.

**Results:** After two and seven days, the immobility time was decreased in the *P. ginseng*-administrated mice as compared to the control group; however, this reduction was not significant. In addition, the amount of TP in the blood serum was significantly increased. However, the levels of Glc, BUN, and LDH did not show a significant change. *P. ginseng* significantly ( $P < 0.05$ ) increased interferon (IFN)- $\gamma$  production and expression as compared to control at 48 h in MOLT-4 cells. *P. ginseng* plus recombinant IFN- $\gamma$  instead of *P. ginseng* alone significantly increased the production of the tumour necrosis factor (TNF)- $\alpha$  in the mouse peritoneal macrophages.

**Interpretation & conclusion:** Our results suggest that *P. ginseng* may be useful for an immune promoter. Further studies are needed to understand the mechanism of its action.

**Key words** Cytokine - forced swimming test - mice - *Panax ginseng*

The Korean herbal medicine, *Panax ginseng* C. A. Mayer (Araliaceae), has been widely used in China and Japan to fight fatigue and for the enhancement of resistance to many diseases<sup>1</sup>. The pharmacological effects of ginseng have been demonstrated in the central nervous system, the cardiovascular system, as well as the endocrine and immune systems. Ginseng and its constituents have been shown to exhibit both anti-stress and anti-oxidant activity<sup>2,3</sup>, and to exert various benefits relating to stress and the immune system<sup>4,7</sup>, and alleged ability to boost the reduced metabolism of weak patients<sup>8</sup>. We have earlier studied the effect of several herbs such as Allergina and Herbkinex on immune response using a forced swimming test (FST) and human lymphocytes<sup>9,10</sup>. Animal experiments and clinical trials have shown that these herbs have immunomodulating activities<sup>11,12</sup>. However, the mechanism of their effectiveness has not been understood.

FST is perhaps one of the most commonly used animal models of behavioural despair, and has been used extensively as a pre-clinical diagnostic tool for the assessment of novel antidepressants<sup>13</sup>. Many psychotropic drugs have been screened with FST<sup>14</sup>. 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 FST<sup>15,16</sup>. Many similar alterations in immune function have been reported in depressed patients<sup>17</sup>. FST has been used as an endurance test and also to examine whether certain agents have anti-fatigue effects<sup>18</sup>.

T-cells play a crucial role in immune functions as they act both as effectors (cytotoxic T-cells, Tc cells) and regulators (helper and suppressor T-cells: Th and Ts cells). The induction of Th immune responses plays a critical role in protecting against various intracellular microorganisms and tumours. Macrophages serve as the first line of defense against

pathogenic microbial insults<sup>19</sup>. Macrophages play an indirect role in these antimicrobial or antitumour activities with their secretion of cytokines (*e.g.*, IL-12) or by antigen processing and presentation, thereby regulating the immune system<sup>20</sup>.

We examined the *Panax ginseng* on forced mobility time and immune factors to know whether this herbal medicine has immuno-promoting ability.

### Material & Methods

*Preparation of P. ginseng:* *P. ginseng* was purchased from Kumsan herbal medicine market (Daejeon, South Korea) and authenticated by Dr S.S. Shin of College of Oriental Medicine, Dong Eui University, South Korea. The root was found to be six years old. The voucher specimen (number 02-03-29) was deposited in the Herbarium of the Department of Oriental Pharmacy, College of Pharmacy, Wonkwang University. No heavy metals were detected from *P. ginseng* in the test by the Korean Oriental Medicine Institute. To obtain an aqueous extract of *P. ginseng*, 102 g of *P. ginseng* was added to distilled water, heat extracted, concentrated with a rotary evaporator and lyophilized. The yield of extraction was about 10 per cent (w/w). The normal dose of many oriental medicines for an adult person is usually around 0.1 g/kg at a time when it is converted into extracted powder<sup>21</sup>. Thus, we screened 3 doses (0.001, 0.01, and 0.1 g/kg) of *P. ginseng*. Maximal effective concentration (0.1 g/kg) has been used in this study.

*Assay for endotoxin determination:* The *P. ginseng* extract used in this experiment was found to be free of endotoxin as determined within the limits of an assay E-TOXATE kit (Sigma, USA), performed according to the manufacturers protocol. In this assay, saturation occurred at 40 EU/ml and the resolution limit was > 0.1 EU/ml.

**Animals:** The original stock of male ICR ddY (ICR) mice (15-20 g) were purchased from the Daehan Biolink Co., (Daejeon, South Korea), and were housed at a temperature of  $23\pm 1^\circ\text{C}$  with a 12 : 12 h light dark cycle. Food and water were available *ad libitum*. The care and treatment of the mice were in accordance with the guidelines established by the Public Health Service Policy on the human care and use of laboratory animals and were approved by the institutional animal care and use committee.

**Forced swimming test (FST)<sup>13</sup>:** Briefly, ICR mice ( $n = 7$ ) were dropped individually into glass cylinders (height: 25 cm, diameter: 10 cm) containing 10 cm of water maintained at  $23\text{-}25^\circ\text{C}$ . Mice remained in the water for 6 min. A 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 *P. ginseng* groups (0.1 g/kg) in such a way as to match the swimming time in each group. Saline/*P. ginseng* was given 24 h before the FST was performed. *P. ginseng* (0.1 g/kg) was orally administered in powder form dissolved in water to mice seven days. We measured the immobility time at the 2 (second FST) and 7 days (third FST) after the administration of saline or *P. ginseng*. In the second and third FST, the mice were allowed to swim for duration of 6 min and observers blind to the drug treatment recorded the immobility times. All of the experiments were carried out between 1000 and 1500 h in testing rooms adjacent to the animal rooms.

**Preparation and ingredient analysis of blood serum:** Changes in several blood biochemical parameters in the mice were measured after the FST. The mice were anaesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (4 mg/kg). After anaesthetization, blood was withdrawn into syringes from the heart. The amounts of serum glucose (Glc),

blood urea nitrogen (BUN), lactate dehydrogenase (LDH), and total protein (TP) were determined by an autoanalyzer (Hitachi 747, Hitachi, Japan).

**MOLT-4 cell culture:** T cell line MOLT-4 cells were grown in a RPMI 1640 medium (Gibco BRL, USA) supplemented with 10 per cent foetal bovine serum (FBS) (JRH BIOSCIENCE, USA), 100 U/ml penicillin and 100  $\mu\text{g/ml}$  streptomycin at  $37^\circ\text{C}$  in the presence of 5 per cent  $\text{CO}_2$ .

**Peritoneal macrophage culture:** Thioglycollate (TG)-elicited macrophages were harvested 3 days after an intraperitoneal injection of 2.5 ml TG to mice. Using 8 ml of Hank's balanced salt solution (HBSS), which contained 10 U/ml heparin, a peritoneal lavage was performed. After this, the cells were distributed in Dulbecco's modified Eagle's medium (DMEM), which was supplemented with 10 per cent heat-inactivated FBS, in 4-well tissue culture plates ( $3 \times 10^5$  cells/well) incubated for 3 h at  $37^\circ\text{C}$  in an atmosphere of 5 per cent  $\text{CO}_2$ . The plates were washed three times with HBSS to remove non-adherent cells, and equilibrated with DMEM containing 10 per cent FBS before treatment. Mouse peritoneal macrophages were cultured with recombinant mouse interferon- $\gamma$  (rIFN- $\gamma$ ) for 6 h and then stimulated with *P. ginseng* (1, 10  $\mu\text{g/ml}$ ) for 24 h.

**3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) assay:** Cell viability was determined by a MTT assay<sup>22</sup>. Briefly, 500  $\mu\text{l}$  of a MOLT-4 cells suspension ( $2.5 \times 10^4$  cells) was cultured in 4-well plates for 24 h after treatment by each concentration of *P. ginseng*. 20  $\mu\text{l}$  of MTT solution (5 mg/ml) was added and they were incubated at  $37^\circ\text{C}$  for an additional 4 h. After the washing out of the supernatant, the insoluble formazan product was dissolved into DMSO. Following this, the optical density of 96-well culture plates was measured using an enzyme-linked immunosorbent assay

(ELISA) reader (CA, USA) set at 540 nm. The optical density of the formazan formed in the untreated control cells was read to be 100 per cent of the viability.

**ELISA assay:** Secreted cytokine levels in culture supernatants of MOLT-4 cells and mouse peritoneal macrophage were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (for IFN- $\gamma$ , IL-4, TNF- $\alpha$  and IL-12 assay, R&D Systems, MN). Optical density was read within 10 min of the addition of the substrate with a 405 nm filter.

**Western blot analysis:** Cell extracts were prepared by a detergent lysis procedure. Cells ( $5 \times 10^6$  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 g for 5 min 4°C. Samples were heated at 95°C for 5 min, and briefly cooled on ice. After the centrifugation at 15,000  $\times$  g for 5 min, 50  $\mu$ l aliquots were resolved by 12 per cent sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Resolved proteins were transferred overnight to nitrocellulose membrane in 25 mM Tris, pH 8.5, 0.2 mM glycerin, 20 per cent methanol at 25 V. Blots were blocked for at least 2 h with 1 x TBST containing 10 per cent nonfat dry milk. Immunodetection was done using an enhanced chemiluminescence detection kit (Amersham).

**Statistical analysis:** The immobility time of *P. ginseng* was evaluated by the Mann-Whitney U-test. Other results were analyzed by the Students- t test. Statistical

analysis of the data was carried out using SPSS v10.0 software. All experiments were repeated at least three times. The values shown are means  $\pm$  SE. The differences were considered statistically with  $P < 0.05$ .

## Results

**Effects of *P. ginseng* on immobility in FST:** The immobility time was decreased in the *P. ginseng*-administrated group (2 day,  $141 \pm 10$  sec; 7 days,  $120 \pm 7$  sec) in a comparison with the saline-administrated group (2 days,  $150 \pm 14$  sec; 7 days,  $140 \pm 9$  sec,  $P = 0.589$ ) (data not shown). However, the difference was not significant.

**Effect of *P. ginseng* on blood biochemical parameters:** When *P. ginseng* (0.1 g/kg) was administered orally to mice, the TP level significantly ( $P < 0.05$ ) increased. BUN and LDH levels also increased but this increase was not significant (Table).

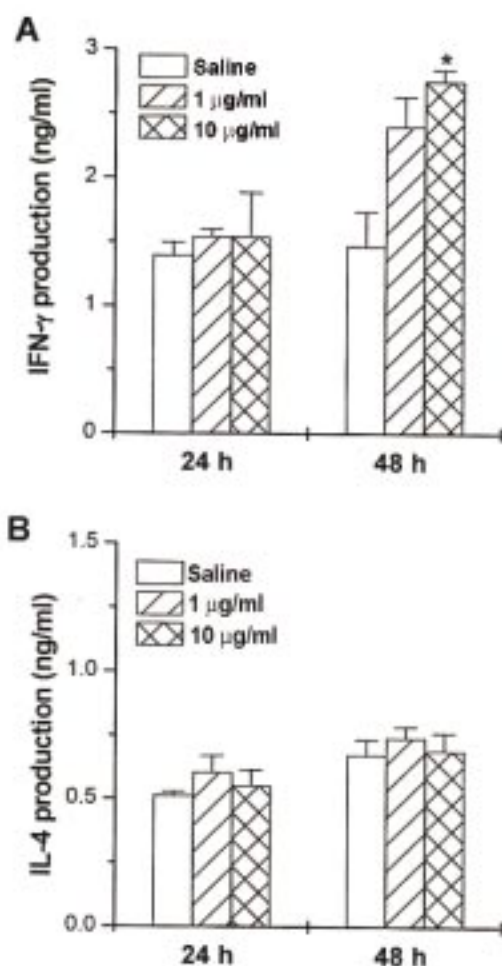
**Effect of *P. ginseng* on the productions of IFN- $\gamma$  and IL-4 on MOLT-4 cell:** *P. ginseng* (10  $\mu$ g/ml) significantly increased IFN- $\gamma$  production compared to the media control (about 2-fold,  $P < 0.05$ ) at 48 h (Fig. 1 A). However, the production of IL-4 was not affected (Fig. 1 B). *P. ginseng* (1, 10  $\mu$ g/ml) also upregulated the IFN- $\gamma$  protein expression compared to the media control (Fig. 1 B).

**Effect of *P. ginseng* on the productions of TNF- $\alpha$  and IL-12 in peritoneal macrophages:** *P. ginseng* alone did not stimulate the production of TNF- $\alpha$ . The

**Table.** Effects of *P. ginseng* on blood biochemical parameters in mice

Treatment	Glucose (mg/dl)	Blood urea nitrogen (mg/dl)	Lactate dehydrogenase (U/l)	Total protein (g/dl)
Saline	$366.0 \pm 9.4$	$18.2 \pm 1.0$	$904.0 \pm 135.3$	$4.9 \pm 0.1$
<i>P. ginseng</i>	$359.0 \pm 18.0$	$22.7 \pm 0.9$	$1113 \pm 88.8$	$5.2 \pm 0.1^*$

Values are mean  $\pm$  SE (N=7). \* $P < 0.05$  compared to saline-administrated group

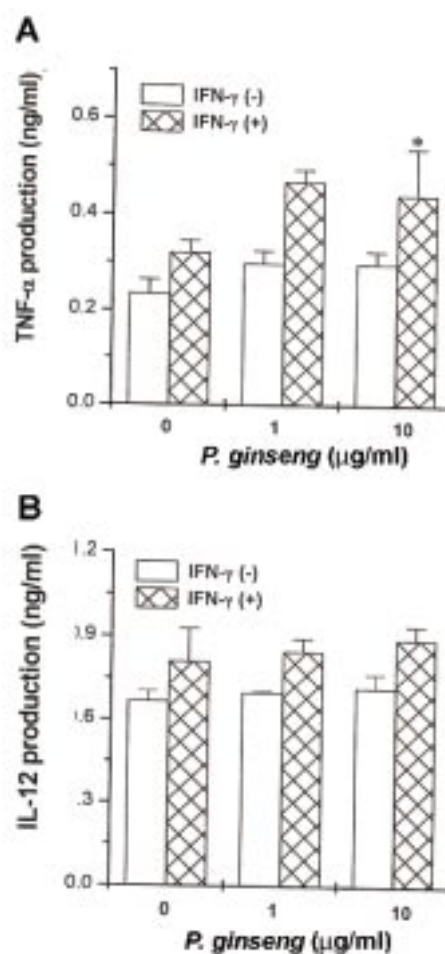


**Fig. 1.** Effects of *P. ginseng* on IFN- $\gamma$  and IL-4 productions in the MOLT-4 cells. Culture supernatant was collected from none or *P. ginseng* treated MOLT-4 cells, which were cultured for 24, 48 h. IFN- $\gamma$  (A) and IL-4 (B) level in culture supernatant was measured using ELISA. Data represent mean  $\pm$  SE of three independent experiments. \* $P$  < 0.05; significantly different from the saline value.

amount of TNF- $\alpha$  was significantly higher in the rIFN- $\gamma$  plus *P. ginseng* treated cells than in the rIFN- $\gamma$  treated cells (about 1.5-fold at the 1  $\mu$ g/ml, and 1.4-fold at the 10  $\mu$ g/ml,  $P$  < 0.05) whereas the IL-12 production was not affected (Fig. 2 B).

### Discussion

The results of our study showed that the duration of immobility in FST was shortened by administration of *P. ginseng*. In general, the



**Fig. 2.** Effects of *P. ginseng* on TNF- $\alpha$  and IL-12 productions in the mouse peritoneal macrophages. Data represent mean  $\pm$  SE of three independent experiments. \* $P$  < 0.05 compared to unstimulated value. \*\* $P$  < 0.05 compared to rIFN- $\gamma$  treated value.

swimming exercise is known to induce biochemical changes in blood<sup>18</sup>. In our study TP level was significantly increased. Protein measurements can reflect nutritional state, kidney disease, liver disease, *etc.* These facts suggest that *P. ginseng* may act as an energy source, and biochemical changes may be related to the decrease in immobility time.

*P. ginseng* is a medicinal plant cultivated in Korea, Japan, China and Russia. The main active constituents of *P. ginseng* are reported to be

saponins, ginsenosides and polysaccharides<sup>23</sup>. Recently, several reports have shown immunomodulating effect of the extract of *P. ginseng*<sup>4,24,25</sup>. Various clinical and pharmacological effects associated with its use have been reported, such as anti-cancer activity, anti-circulatory shock effects, the promotion of haematopoiesis, and the modulation of immune functions and cellular metabolic processes on carbohydrates, fats and proteins<sup>26-28</sup>. *Ginseng* extract has been shown to have antihypertensive, antidiabetic and antinociception effects<sup>29-31</sup>. Shah *et al* reported that Korean ginseng tea has cerebroprotective effects in global and focal models of ischaemia in rats<sup>32</sup>.

Immunoregulatory cytokines play an important role in determining the nature and strength of an immune response<sup>33</sup>. Previous studies have demonstrated that the induction of Th1-promoting cytokine, using specific adjuvants, can enhance anti-tumour immunity and can reduce or even prevent tumour growth<sup>34</sup>. 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- $\gamma$  and TNF- $\alpha$ <sup>35</sup>. IFN- $\gamma$  is an important cytokine in the host defense against infection by viral and microbial pathogens<sup>36</sup>. It induces a variety of physiologically significant responses that contribute to immunity. TNF- $\alpha$  participates in host defense against pathogens. TNF- $\alpha$  modulates the immune response by triggering the production of a number of other regulatory cytokines<sup>37</sup>. The present results indicated at the association of *P. ginseng* with the Th-1 immune response. TNF- $\alpha$  production in mouse peritoneal macrophages by *P. ginseng* may be highly stimulated in combination with rIFN- $\gamma$ . These results suggest that *P. ginseng* may provide a second signal for the synergistic induction of TNF- $\alpha$ .

In conclusion, *P. ginseng* decreased the immobility time in FST in mice. *P. ginseng* also induced the production of IFN- $\gamma$  from MOLT-4 cells and TNF- $\alpha$  from mouse peritoneal macrophage. These results suggest that *P. ginseng* may have beneficial effects on immune-enhancement. Further studies will be needed to elucidate whether the increased cytokine production in this experimental model is directly related to the immobility behaviour.

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