原　著

Effect of *Ganoderma lucidum* on radioprotection in Mice

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Key Words: *Ganoderma lucidum*, Flow cytometry, Humoral immunity, Immunocompetence, CD4, CD8.

Abstract

The characteristic constituents of *Ganoderma lucidum*, such as polysaccharides, triterpenoids, nucleic acids, and small proteins, have been found and proved to have many special pharmacological properties. Mice have been used to extensively investigate the effects of *G. lucidum*. Experiments with mice investigating the effects of concentrates of *G. lucidum* have never been reported. The purpose of this investigation was to understand the effect of *G. lucidum* feeding on blood biochemistry and immunocompetence in mice. Complete blood count (CBC) and blood biochemistry were surveyed routinely. Cellular-mediated immunity was monitored using flow cytometry to survey the percentage changes in $CD3^+$, $CD4^+$, and $CD8^+$ T-lymphocytes and B-lymphocytes in the peripheral blood lymphocytes (PBL). The effect of *G. lucidum* on humoral immunity was examined with a fast plate agglutination test to know the manifestation and change in the titer of specific anti-egg albumin antibodies in the serum after egg albumin injection. The findings on CBC and blood biochemistry indicated that *G. lucidum* was

quite safe for mice. Experimental results on cell-mediated immunity showed that *G. lucidum* could increase the percentage of $CD3^+$, $CD4^+$, and $CD8^+$ T-lymphocytes in PBL. Experimental results on humoral immunity showed that *G. lucidum* could help mice to produce a significantly higher quantity of specific antibodies in a shorter time.

Introduction

We have found that *Ganoderma lucidum* has a higher activity than species of the same class of shiitake regarding anti-tumor activity and made clear that the acid β-1,6 and β-1,3 D-glucans, and their active bodies β-1,6 and β-1,3 D-glucans are contained in *G. lucidum*[2]. The fungus *G. lucidum* has been used as a folk medicine in the orient and has recently attracted much attention due to its biological activities[1]. Investigations on constituents of the carpophores and the cultured mycelia of *G. lucidum* have shown that this mushroom has various biological activities. *G. lucidum* was reported to contain some intensely bitter compounds including lucidenic acid A, B, C, D, E, lucidone A, and ganoderic acids B, which are known to inhibit both histamine release from mast cells[2] and an angiotensin converting enzyme that is responsible for hypertension and growth of liver cancer cells[3,4]. *G. lucidum* was also reported to contain polysaccharides and protein-bound polysaccharides that have anti-tumor properties, antihypertensive activities, and also causes decrease in blood glucose level[5,6,7,8]. These components can also modulate and enhance immunity and stimulate specific immunocytes to produce interleukins, interferons, and tumor necrosis factors (TNFs)[9,10].

Most investigations on *G. lucidum* were performed with rodents as experimental animals or with *in vitro* experiments. There is no evidence to prove that *G. lucidum* is also effective in mice that are sloppy appetite with a large cecum and monogastric digestive tract. No enough information regarding the oral administration of *G. lucidum*, its mechanism of absorption, and its effect by digestive enzymes and enteric micro flora is available.

The present experiment provides information regarding the oral feeding of *G. lucidum* and its effects on blood chemistry and immunocompetence in mice.

Materials and Methods

Experimental animals

Five-week-old male ICR [Crj: CD-1 (Swiss Hanchka)] mice with an average weight of 18-20 grams were purchased from Japan SLC Inc. and kept under standard conditions (room temperature 22 ± 3 ℃, humidity 60%) and consistent feeding (CA-1, Japan Clare, Inc.) and drinking water (tap water). The mice were acclimated to the breeding and experimental environment for one week prior to the experiments. The experimental animals used for the studies on IgG and IgM in peripheral blood were six-week old male C3H/HeNCrj mice (average body weight, 18-24 g) purchased from Charles River, Japan. For the studies on T-lymphocytes, three-week old male C57BL/6crSlc mice (body weight, 8-13 g on average) purchased from Japan SLC Inc. were used.

Administration of *G. lucidum*

G. lucidum used for this study was obtained from Cress Pham. Ltd. *G. lucidum* liquid extract was orally administered to the mice at a dose of 400 mg/kg every other day. After administration of G. lucidum for at least three weeks, mice were used for the experiments and the *G. lucidum* administration was continued until the end of the experiment.

Measurement of peripheral blood cell counts in mice

Changes in peripheral blood cell counts were studied in 2 groups of mice: a control group, which was administered saline; an *G. lucidum* only group, which was administered *G. lucidum* only group. Ten mice for each group were used in the experiment.

The tail vein of each mouse was cut with a Spitz

knife and 10 μl of peripheral blood was collected with a capillary tube. The blood cell count was measured with an automated blood cell counter (Celltac-α MEK-6318, Nippon Koden Inc.). In particular, the numbers of peripheral leukocytes, lymphocytes, granulocytes, and monocytes, all of which have relatively high sensitivity to radiation, and major cells of the immune system were counted. In order to observe changes in the peripheral blood cell counts, a series of measurements was done; the preceding day of irradiation, and at 3 hours, 12 hours, 24 hours, 3 days, 7 days, 15 days, and 30 days after irradiation. Statistical analysis was performed using the parametric ANOVA (analysis of variance) test among the groups to determine significant differences ($P < 0.05$) in blood cell counts.

Blood Analysis

Whole blood was collected from the mouse heart by puncturing with a 23-G needle under anesthesia, mixed with heparin, and either centrifuged (15 min at 1,500 rpm) to separate serum for the immunoglobulin studies, or suspended 1:1 with phosphate buffered saline (PBS) and then processed as described below for the T-lymphocyte studies.

Measurement of CD3-, CD4-, and CD8-positive T-lymphocytes in peripheral blood

Lymphocytes were separated by the gravity centrifugation method[7]. Lymphocyte separating solution (5 mL; sodium hypaque, Ficoll 400; specific gravity, 1.0875 ± 0.0005 at 25℃) was placed into a 15-ml sample tube, on to which 5 ml of cell suspension was carefully loaded. After centrifugation at 500g for 20 min at room temperature (15-20℃), plasma in the supernatant was collected to extract lymphocyte subsets. After addition of PBS (pH 7.2, without Ca^{2+} and Mg^{2+}) supplemented with 10% inactivated FBS (fetal bovine serum; heat-inactivated at 56℃ for 30 min), and red blood cell lysing solution, the mixture was centrifuged at 400g for 10 min at room temperature. The supernatant was collected and the cells were resuspended and washed twice with PBS containing FBS. Lymphocytes were resuspended in PBS prior to analysis.

Flow cytometry reagents for lymphocyte subset measurement were added to the lymphocyte suspension in PBS, and the mixture was stained for immunofluorescence for about 30 min at 4℃ in a dark room. After the reaction, the solution was rinsed three times with PBS, and CD3, CD4, and CD8 subsets were analyzed by a FACS Caliber flow cytometer (Becton Dickinson). To analyze T-lymphocyte subsets, a Multicolor Flowcytometry (FCM) System (Santa Cruz Biotechnology Inc.) was employed and CD3-, CD4-, and CD8-positive T-lymphocytes in the peripheral blood were counted by three-color flow cytometry using anti CD3-PE- Cy5.5 (Phycoerythrin Cy5.5), anti CD4-FITC (fluorescein isothiocyanate), and anti CD8-PE (Phycoerythrin).

The animal experiment in this study obtained approval of the Ethical Review Board of our university.

Results

Changes in mouse peripheral blood cell counts

Fig. 1 shows time-dependent changes in leukocyte number in each group. The *G. lucidum* group showed a significant increase in leukocyte cell number, as compared to the control group. The days with increased leukocytes were 1, 3, 7, 15, and 30 days of the *G. lucidum* group, which were the whole period after the treatment and the extents of the increases were all significant. These results demonstrate that *G. lucidum* definitively increase the number of leuko-

cytes.

Lymphocytes

Fig. 2 shows the time-dependent change in the number of lymphocytes in each group.

The *G. lucidum* group showed a high lymphocyte number as compared with the control group ; statistically significant differences were observed in many periods ($P < 0.05$). Thus, a tendency to increase the number of lymphocytes was demonstrated in the *G. lucidum* groups. With respect to time-dependent change after dose, all the experimental groups

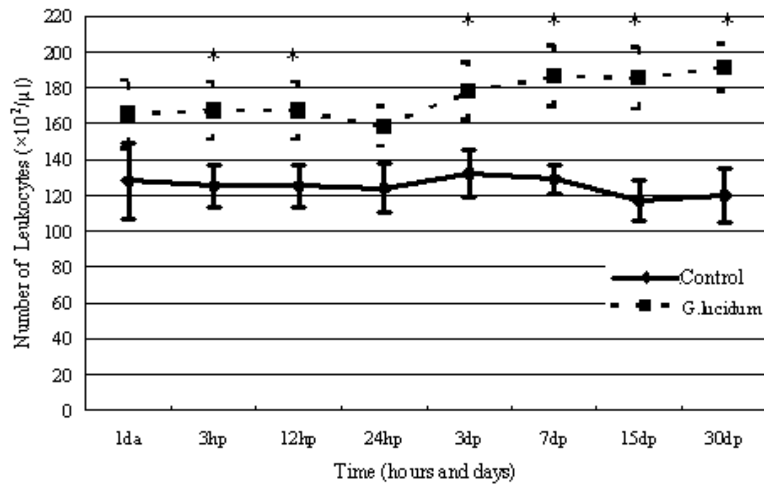

Fig. 1. Shows time-dependent changes in leukocyte number in each group. Significantly different from $^{*}P < 0.05$ Control group vs. G. lucidum groups by ANOVA test. (da ; days after, hp ; hours post, dp ; days after)

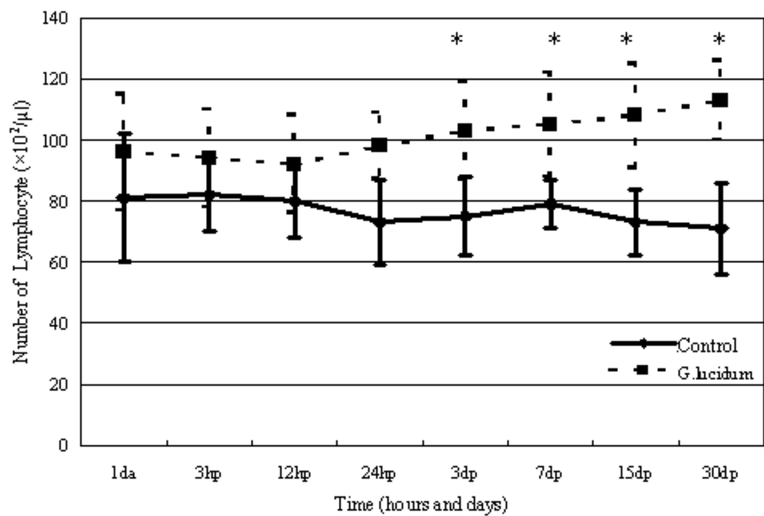

Fig. 2. Shows time-dependent changes in leukocyte number in each group. Significantly different from $^{*}P < 0.05$ Control group vs. G. lucidum groups by ANOVA test. (da ; days after, hp ; hours post, dp ; days after)

showed a decrease and subsequent restoration was observed 1, 3, 7, 15, and 30 days after *G. lucidum* administration.

Analysis of T-lymphocyte subsets in mouse peripheral blood

The lymphocyte fraction was injected into a cytographic analyzer, and CD4-positive ($CD3^+$ $CD4^+$) and CD8-positive ($CD3^+$ $CD8^+$) cells were counted. CD4-positive cells were first counted. As shown in fig. 3 shows, the number of CD4-positive cells increased by 40% on 7 days after *G. lucidum* administration. On 10 days after administration, the number of CD4-positive cells increased by 95%. These results demonstrate that *G. lucidum* administration increases peripheral blood CD4-positive cells, i.e., helper T cells.

CD8-positive cells were also counted by flow cytometry. As fig. 4 shows, CD8-positive cells increased by 81% in the *G. lucidum* group on 7 days after the administration when compared with the control group. In contrast to the *G. lucidum* group, which were significantly increased throughout the whole period of treatment, CD8-positive cells on 7 days of the control group were decreased by 85%. These results clearly indicated that *G. lucidum* administration increases peripheral blood CD8-positive cells, i.e., suppresser T cells and killer T cells. However, in the *G. lucidum*, irradiation decreased the number of CD8-positive cells.

Discussion

In this study, we investigated effects of *G. lucidum* on the blood chemistry and immunocompetence in mice. In the first experiment, we surveyed the complete blood count and blood biochemistry of the mice. All surveyed items of 10 mice in three groups ranged in their reference values. Cell-mediated immunity showed that *G. lucidum* could increase the percentage of $CD3^+$, $CD4^+$, and $CD8^+$ T-lymphocytes in PBL ($P < 0.05$). Regarding humoral immunity, it was shown that *G. lucidum* could help mice to produce a significantly higher quantity of specific antibodies in a shorter time when foreign pathogenic antigens invaded ($P < 0.05$). The range of curative effects of *G. lucidum* has already been investigated in animals such as mice, rats, rabbits and even human beings[9]. However, there is no evidence to prove

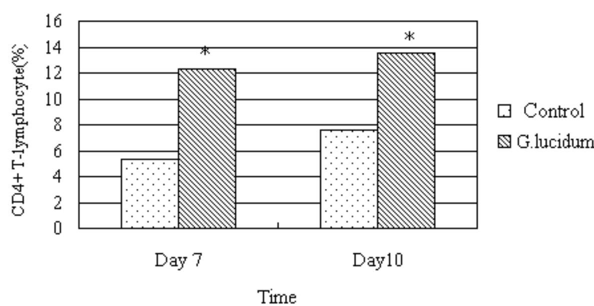

Fig. 3. The increased percentage of $CD4^+$ T-lymphocytes in PBLs compared to the experimental data baselines of the groups. The unit is in percentage (%). Significantly different from $^*P < 0.05$ Control group vs. *G. lucidum* groups by Dunnett test.

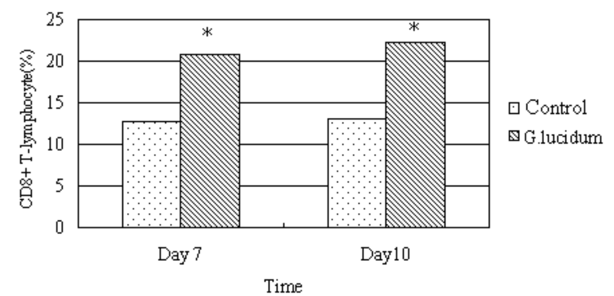

Fig. 4. The increased percentage of $CD8^+$ T-lymphocytes in PBLs compared to the experimental data baselines of the groups. The unit is in percentage (%). Significantly different from $^*P < 0.05$ Control group vs. *G. lucidum* groups by Dunnett test.

whether a liquid extract of *G. lucidum* is also effective or for mice.

In this study, we found that a liquid extract of *G. lucidum* is also effective for mice, and that the extract can enhance the immunocompetence of mice and is safe in using in regulated doses. Its effects on both cellular-mediated and humoral immunity can stimulate the resistance of mice to invading pathogens. Up to the present, there has still been no clear understanding of the metabolic route and action mechanism of the constituents of *G. lucidum*. Questions on how and where *G. lucidum* enters the body via the digestive system to induce such effects remain.

Our previous experimental results have demonstrated that the biologically active components of the cultured mycelia of *G. lucidum* are not affected and destroyed by normal floras in the cecum or by digestive enzymes[11]. There is a possibility of the absorbing sites of *G. lucidum* that locate in the anterior alimentary canal before the cecum. However, our experimental results presented herein could provide some clues to unlocking the mystery of the pathway, the mechanism of how *G. lucidum* is absorbed, and the biological effects observed in mice. *G. lucidum* is speculated to have actions that macrophages are activated *via* stimulation of the intestinal immune system, the activated macrophages release TNF-α, helper T cells are then activated, and the systemic immune system, consisting of macrophages, cytotoxic T cells, killer T cells, NK cells, and B cells is finally activated[12,13,14,15,16].

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