

Full Length Research Paper

A non-toxic herbal remedy which enhance lymphocyte activity and cytokine secretion: *Ganoderma lucidum*

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Ganoderma lucidum is grown in the deep foliage of South-western China, where it is free from heavy metals and pollutions from populated cities. *G. lucidum* contains biologically active components, polysaccharides, which have been shown to enhance the immune system. In China, *G. lucidum* is commonly used to treat cancer, allergies, inflammation, hypertension and platelet-aggregation. This study investigates the proliferation, metabolism and cytokine secretion of lymphocytes *in vitro* upon *G. lucidum* stimulation. The powder of *G. lucidum* was provided by Mei Shan Tang Limited (Hong Kong) and tested to be free of heavy metals and microorganisms. Lymphocytes were extracted from 66 healthy human subjects and cultured with a pre-determined optimal concentration of *G. lucidum* (50-100 ug/mL) or a control medium for 72 h. Proliferation was measured with cell proliferation reagent, and metabolic rate was measured with ATP bioluminescence assay. Levels of IL-2, IL-4, IL-8, IL-10, GM-CSF and TNF- α were measured with ELISA. Meanwhile, toxicity was tested by feeding 70 rats with *G. lucidum*. No significant toxicity was detected in the rats. In human lymphocytes, *G. lucidum* was found to significantly increase cell proliferation and metabolism ($p < 0.05$). T lymphocytes also demonstrated aggregation after *G. lucidum* treatment, which indicated T lymphocyte transformation. *G. lucidum*-treated cells also demonstrated a significantly increased secretion of IL-2, IL-8 and TNF- α ($p < 0.05$). *G. lucidum* has the ability to enhance lymphocyte proliferation and metabolism without any significant toxicity *in vitro*. It also increases the secretion of cytokines, thus suggesting the potential of enhancing cellular immunity. *G. lucidum* is therefore potential candidate as a safe immunostimulant for patients on anti-neoplastic therapy.

Key words: *Ganoderma lucidum*, immune system, cell proliferation, cell metabolism, toxicity, cytokines.

INTRODUCTION

Ganoderma lucidum (*G. lucidum*: Lingzhi in Chinese, Reishi in Japanese) has been considered and being used as a traditional Chinese medicine for thousand years. Lingzhi, which was first mentioned in Shen Nong Ben Cao Jing (dated in 206 BC to 8 AD) (Lu et al., 2004), was considered to be a mushroom that could promote health, vitality and longevity without toxicity (Hsu et al., 2004; Lien, 1990a; Shiao et al., 1994; Wang, 2002). However,

the mechanisms to improve health and longevity were all unknown in ancient years. Nowadays, the Chinese are still consuming Lingzhi and it raises interests for scientists to discover its thousand-year-long myth.

Lingzhi is a mushroom which is now rarely found naturally in deep foliage of remote, wild areas in South-western China. The fruit bodies of *G. lucidum* has been extracted and investigated for its chemical ingredients. Researches have found that the fruiting bodies, spores and mycelia of *G. lucidum* contain several bioactive substances such as polysaccharides, proteins, fatty acids, nucleic acids, sterols and bioinactive substances such as water and alcohol. Among these substances, polysaccha-

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rides such as beta-D glycan (Kuo et al., 2006), are found to be the most important substance in Lingzhi for health promotion (Chung et al., 2001; Hsu et al., 2004; Wong et al., 2004). Polysaccharides and alcohol have both immuno-modulating and anti-tumor effects that inhibit tumor growth (Cao and Lin, 2006; Hsu et al., 2004; Lin, 2005). Immuno-modulating effect results due to the presence of polysaccharides, which induce polysaccharide-mediated immune system for the production of several of cytokines such as IL-1, IL-6, and TNF- α from activated macrophages and IFN- γ from activated T-lymphocytes (Hsu et al., 2004). It also promotes the activities of antigen-presenting cells and phagocyte system (Lin, 2005). Moreover, its anti-tumor effect is due to anti-angiogenesis of tumor cells. Although the mechanism remains to be determined, these results were observed in animals with tumor consuming Lingzhi.

In our study, cell metabolism, proliferation and cytokine secretion, IL-2, IL-4, IL-8, IL-10, GM-CSF and TNF- α of lymphocytes were measured upon stimulation of *G. lucidum* with ATP bioluminescence assay, WST-1 reagent assay and ELISA, respectively.

MATERIALS AND METHODS

Lymphocytes isolation and cell culture

66 healthy adult donors without systemic diseases were recruited from Hong Kong Red Cross by consecutive sampling on a randomized base. Heparinised peripheral blood was collected from them after their consent. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Paque (Amersham Biosciences, Sweden) (Lotze and Rosenberg, 1981). The lymphocytes were isolated and cultured in standard conditions (Chow et al., 2001).

Preparation of *G. lucidum* *G. lucidum* (Mei Shan Tang, Hong Kong) was tested by SGS Hong Kong Limited (Société Générale de Surveillance) to have heavy metals within the international safety level and free from microorganisms. The powder was dissolved in Hank's Balanced Salt Solution (HBSS, Invitrogen, USA) to a concentration of 100 mg/ml and was stored at room temperature. It was then diluted in HBSS and filtered using 0.22 μ M Cellulose Acetate (Corning, USA) for sterilization.

Cell proliferation assay on lymphocytes under the stimulation of *G. lucidum*

Before the measurement of cell proliferation of lymphocytes under the stimulation of *G. lucidum*, a pre-determined optimal concentration of 10 μ g/mL *G. lucidum* was determined and used for the medium. For test groups, lymphocytes were cultured and incubated into a 96-well plate (Nuclon, Denmark) for 72 h after in the presence or absence of *G. lucidum* stimulation. For positive control group, phytohaemagglutinin (PHA), a mitogenic lectin, 20 μ g/mL, was added to the medium (Mills et al., 1985; Wang et al., 2002; Zhao et al., 2005). At the harvest day, 10 μ L of WST-1 reagent (Roche, Germany) was added and incubated with the stimulated lymphocytes according to the instruction provided by the manufacturer. The optical density read was 450 nm by the Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) such that the optical density was directly proportional to the cell proliferation. Metabolism and aggregation of lymphocytes stimulated by *G. lucidum*.

Lymphocytes metabolic rates were measured in the presence or absence of *G. lucidum* using ATP bioluminescence assay. At the harvest day, the cells were centrifuged and transferred to eppendorf tubes and 0.3 ml cell lysis reagent was added. Cell lysis was accelerated by a sonicator (Sonics and Materials Inc. Danbury, CT, USA) at a pulse of 30 per min with 5% amplitude to enhance the release of ATP from cells. A 10-s binding period was proceeded by amalgamation of 50 μ L of both samples and luciferase reagent. The absorbance values of the samples were read by TD-20/20 Luminometer (Turner Designs, CA, USA) at 420 nm wavelength. This kit provides a standard ATP for conversion of samples' optical densities to bioluminescence (RLU, Relative Light Units). Besides, lymphocytes were examined under the microscope to observe aggregations with the application of *G. lucidum*, PHA and with no application.

Enzyme-linked immunosorbent assay (ELISA)

For ELISA analyses of cytokines, a standard curve was first prepared using known concentrations of recombination cytokines. Then, PBLs (2×10^6 cells/ml) were treated and incubated with *G. lucidum* or PHA or nothing for 72 h. Cytokine levels of IL-2, IL-4, IL-8, IL-10, GM-CSF, and TNF- α in culture supernatants were measured with ELISA. Procedures were followed according to the instruction from the ELISA kits (R and D, Minneapolis, USA). The concentrations of cytokines in each sample were measured from the standard curve.

Toxicity of *G. lucidum* to animals

To test for the toxicity of *G. lucidum* on animals, two groups of rats (70 rats in each group) were treated with and without *G. lucidum*. Mean body weight was then measured every day and compared to investigate the toxicity of *G. lucidum* in terms of body weight. The dose of *G. lucidum* treated to rats was calculated as same as the ratio of human weight to dose of *G. lucidum*.

RESULTS

ATP bioluminescence assay, proliferation rate and aggregation of lymphocytes

Two assays on lymphocytes in the presence *G. lucidum* showed that lymphocytes had increased in cell proliferation and metabolic rate. From ATP bioluminescence assay, it was 545 rlu (Table 2) and from the WST-1 reagent assay, it was 0.582 (Table 2). Furthermore, using microscope, aggregation of lymphocytes observed was the largest in the presence of PHA and the lowest with no application of PHA or *G. lucidum*. For the application of *G. lucidum*, mild aggregation of lymphocytes was observed.

Production of cytokines from lymphocytes under the stimulation of *G. lucidum*

From the result of ELISA, IL-2, IL-4, and TNF- α had no significant differences in concentration between the stimulation of *G. lucidum* and the control group while IL-8,

Table 1. Rats body weight with or without TGL.

Group	Mean weight(g)	Minimum(g)	Maximum weight (g)
Control	246.5000	195.00	310.00
TGL	255.9583	196.00	304.00

Table 2. The ATP released and proliferation rate of lymphocytes under the normal condition, the presence of PHA and the presence of TGL.

Group	Mean of ATP(rlu)	Mean of proliferation Rate(WST-1)
Control	453	0.546
PHA	672	0.606
TGL	545	0.582

Table 3. The production of IL-8 with the stimulation of PHA or TGL.

	Mean	Std. Deviation	Minimum	Maximum
Control	176.954182	5.6285187	165.7212	185.2404
PHA	*178.134615	17.5461521	116.8750	192.2596
TGL	*182.254558	6.8347787	170.4327	193.9904

Table 4. The production of IL-10 with the stimulation of PHA or TGL.

	Mean	Std. Deviation	Minimum	Maximum
Control	9.541077	6.2854557	4.7627	24.3390
PHA	*168.823458	22.9776183	97.8983	182.1356
TGL	*50.902936	50.3047145	14.5085	182.7288

IL-10, and GM-SCF (Tables 3-5, Figures 1-3) showed increment of concentration under the stimulation of *G. lucidum*.

***G. lucidum* toxicity to rats**

All 70 rats in both groups are physically and mentally healthy. The mean body weight of the group treated with *G. lucidum* was 256 g while that of the group with the absence of *G. lucidum* was 247 g (Table 1). The mean weight of the group treated with *G. lucidum* was slightly heavier than that of the group in the absence of *G. lucidum*.

DISCUSSION

Lymphocytes under the stimulation of PHA always show a high increment in secretions of cytokines (Katial et al., 1998) as PHA is a mitogen that triggers cell division of T-lymphocytes, and activates peripheral lymphocytes (Tsai and Liu, 1992). It acts as a reference point to our study that the stimulation of *G. lucidum*, on lymphocytes,

should be less than, or at least not largely more than the stimulation of PHA. Otherwise, it will elicit an acute immune response, which is harmful and is not beneficial to patients when used clinically. According to our study, we showed that *G. lucidum* increased lymphocyte proliferation, metabolism and cytokines productions, such as IL-8, IL-10 and GM-CSF (Tables 3-5, Figures 1-3). These indicated that lymphocytes are more active under the stimulation of *G. lucidum in vivo* and were consistent with the research that *G. lucidum* enhances lymphocyte proliferation (Zhu and Lin, 2005). Activating lymphocytes are beneficial as *G. lucidum* stimulates the production of new lymphocytes; the largely suppressed immune response system of breast cancer patients was boosted up slightly during radiotherapy and chemotherapy. Although the increments of magnitudes of cytokines productions are different under the stimulation of *G. lucidum*, the mushroom can be useful in enhancing the immune response *in vivo*. Taking *G. lucidum* can mildly increase the cytokines production by lymphocytes without harming the body.

IL-8, IL-10 and GM-CSF, which were stimulated by TGL (Figures 1-3) have different effects on human bodies. IL-

Table 5. The production of GM-CSF with the stimulation of PHA or TGL.

	Mean	Std. Deviation	Minimum	Maximum
Control	0.229692	.1475587	.1050	.5440
PHA	0.355467	.3257038	.1030	1.2500
TGL	*1.071000	.2508785	.8060	1.6020

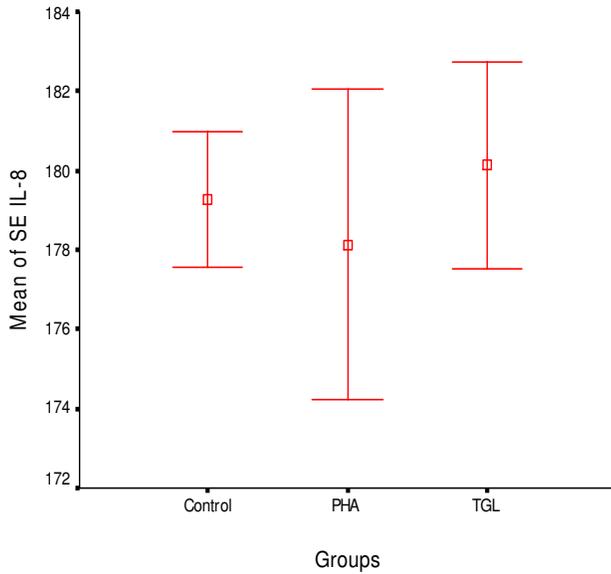


Figure 1. Graph showing the mean secretion of IL-8 under the stimulation of PHA or TGL compare with the control group.

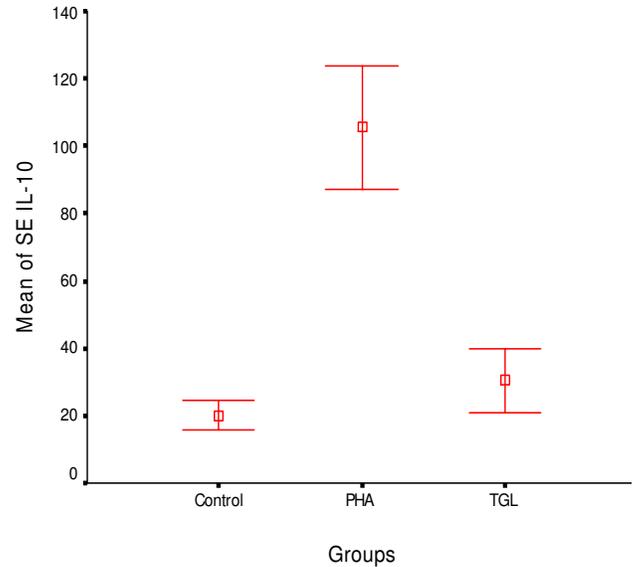


Figure 2. Graph showing the mean secretion of IL-10 under the stimulation of PHA or TGL compare with the control group.

8, a proinflammatory interleukin, is produced when exposed to inflammatory stimuli such as IL-1 or tumor necrosis factor. This interleukin activates neutrophils inducing chemotaxis, exocytosis (Baggiolini and Clark-Lewis, 1992) GM-CSF, also a proinflammatory interleukin, is mainly secreted by T-cells and stimulates the production of granulocytes and monocytes. Monocytes then migrate to different tissues and eventually differentiate into macrophages.

This brings inflammatory responses but at the same time, it enhances the production of granulocytes and macrophages to fight infection by pathogens. Pro-inflammatory cytokines are important for host survival from the infection by bacteria or virus and is required for the repair of tissue injury. These beneficial effects, however, are critically dependent on the magnitude of immune response, because large amounts of inflammatory mediators can also cause collateral damage to normal cells (Shiao et al., 1994). *G. lucidum* is good at partially increasing the production of pro-inflammatory cytokines such that it will not bring damage to normal cells. IL-10, in contrast, is an anti-inflammatory interleukin which suppresses the synthesis of pro-inflammatory cytokines (Girndt, 2002). It plays an important role in regulating inflammatory and immune reactions (Beebe et

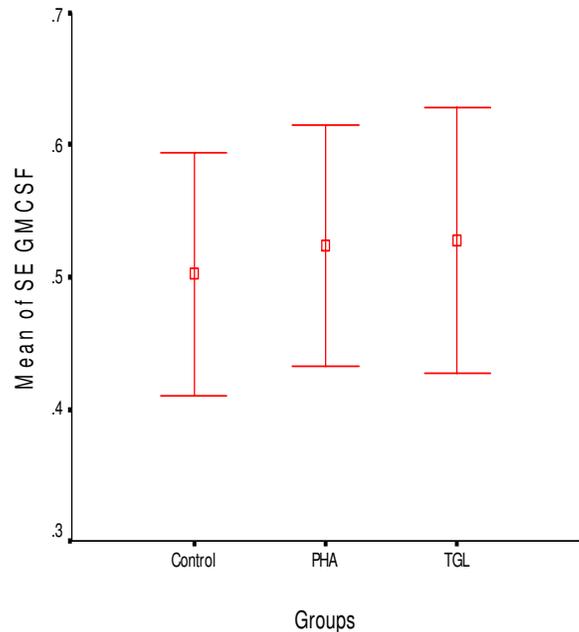


Figure 3. Graph showing the mean secretion of GM-CSF under the stimulation of PHA or TGL compare with the control group.

al., 2002). Not only can *G. lucidum* enhance patient's immune system, it was found that it has the activity of liver protection, hypoglycemia and platelet-aggregation inhibition (Chen et al., 2004; Hsieh and Wu, 2002; Lien, 1990b; Lu, 2001, 2002).

In summary, *Takayama Ganoderma Lucidum* enhances the proliferation, metabolism and cytokine productions of IL-8, IL-10 and GM-CSF without bringing observable harmful effects and could potentially be used to treat various kinds of diseases such as bacterial infections.

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