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Effect of *Ganoderma lucidum* on Cytokine Release by Peritoneal Macrophages

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ABSTRACT

Background: The water-soluble extract of *Ganoderma lucidum* (Reishi) has been used as an immunomodulator to stimulate spleen cells proliferation and cytokine expression. **Objective:** To investigate the effect of *Ganoderma lucidum* (G. lucidum) on cytokine production by mice peritoneal macrophages. **Methods:** Mice peritoneal macrophages were prepared by intra-peritoneal injection of 5 ml cold PBS. Peritoneal macrophages were plated out at $1X10^6$ cell/well in 1ml RPMI 1640 medium supplemented with 10%FCS, 50 μg streptomycin and 50U penicillin. Cells were incubated in the presence or absence of different concentrations of G. lucidum at 37^0C and 5% CO_2 for 48 hours. Cell free medium was removed and used for cytokine assay by ELISA method (Bender med system). **Results:** The results showed no significant differences in cell viability at concentrations ranged from 0-40 μg/ml compared with control group. G. lucidum enhanced IL-1β, TNF-α and NO production in a concentration dependent manner. However, it is not clear if the enhancement of NO release is due to direct effect of G. lucidum on NO synthesis or by indirect endogenous modulation via cytokines. IL-12 release by peritoneal macrophages was also increased in response to different concentrations of G. lucidum, but maximum enhancement was induced in response to 5 μg/ml of G. lucidum ($p<0.001$). **Conclusion:** Our results indicate that G. lucidum at concentrations used has a positive effect on cytokine release and NO production by peritoneal macrophages. Therefore, it is concluded that G. lucidum at moderate concentrations improves macrophage function through cytokine and NO release.

Keywords: Macrophage, Ganoderma lucidum, Interleukin-1, Interleukin-12, Tumor Necrosis Factor and Nitric Oxide

INTRODUCTION

Ganoderma lucidum is a basidiomycete white root fungus which has been used for medicinal purposes for centuries in China, Japan and Korea (1). It has also been investigated as anti-tumor and antiviral agent and less so as anti-bacterial agent (2).

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Different components of G. lucidum modulate unrelated signalling and therefore it can possess synergistic effects (3). Thus, triterpene compounds in G. lucidum suppress growth and invasive behaviour of cancer cells, whereas its polysaccharides stimulate the immune system resulting in the production of cytokines and anti-cancer activation of immune cells (4). G. lucidum has also been shown to have an immuno-modulating effect in patients with advanced colorectal cancer (5).

Some investigators believe that anti-tumor activity of G. lucidum might be due to its nitric oxide (NO) modulation by immune cells (6). They demonstrated that G. lucidum significantly inhibited LPS-induced NO production in RAW 264.7 macrophages. On the other hand, Nonaka et al. suggested that anti tumor activity of G. lucidum is due to high content of β-D glucan in its fruiting body and pointed out that one of the anti tumor mechanisms of G. lucidum is due to its IFN-γ inducible activity and cytotoxic T lymphocyte activation under the cancerous condition (7).

It has also been reported that when human umbilical cord blood mono nuclear cells were treated with the polysaccharides of G. lucidum (10-100μg/ml) for 7 days, the population of CD14+ CD26+ monocyte/macrophage, CD83+ CD1a+ dendritic cells and CD16+ CD56+ NK cells were 2.9, 2.3, and 1.5 times higher than those of untreated control (8). It has also been demonstrated that the polysaccharides isolated from G. lucidum enhanced the cytotoxic activity of NK cells, secretion of tumor necrosis factor-α (TNF-α) and interferon γ (IFN-γ) (8), phogocytosis and cytotoxicity of macrophages (9).

In advanced colorectal cancer, treatment with G. lucidum has been shown to increase mitogenic reactivity to phytohemagglutinin, counts of CD3, CD4, CD8 and CD56 lymphocytes, plasma concentration of IL-2, IL-6, IFN-γ and NK cell activity and decrease plasma concentration of IL-1 and TNF-α (5). On the other hand, it was demonstrated that oral administration of 500 mg/kg of G. lucidum enhanced IFN-γ and IL-12 production (10).

In a recent report it was illustrated that G. lucidum also stimulates TNF-α, and IL-6 release after 8 hour treatment of human whole blood (11). The effect of G. lucidum on cytokine production by mouse spleen cells, human macrophage cell line (J774A.1) and murine macrophage cells (EORP) has also been reported (12,13).

Meanwhile, the effect of G. lucidum on cytokine production by mice peritoneal macrophages has not yet been reported. Therefore, the aim of this study was to find out the effect of G. lucidum on cytokine release by resting peritoneal macrophages of mice.

MATERIALS AND METHODS

Eight week old BALBc male mice were maintained in dust free bedding cages in the animal unit. Twelve animals were anesthetized and subjected to 5 ml ice cold PBS intraperitoneally (IP). Then, the peritoneal cells were extracted immediately and kept at 2-8^{0}C to avoid non-specific attachment prior to washing for 3 times by centrifugation at 1500g for 5 min using cold RPMI 1640 (Sigma Chemical Co). Then the cells were resuspended in RPMI 1640 supplemented with 10% FCS, 50 μg streptomycin and 50U penicillin (Sigma Chemical Co) and counted. Cell viability was checked by trypan blue exclusion test. An aliquot of the cell suspension was mixed with an equal volume of 0.4% (W/V) Trypan blue in PBS and incubated for 10 minutes. The cells failing to exclude the dye were counted and expressed as a percentage of the total cells present. Cell viability= (number of viable cells) / (sum of viable and dead cells) X 100 (14).

Cell Culture. The cells were plated out at $1x10^6$cells/well in 24 well plates and allowed to adhere for two hours (n=6). Non-adherent cells were then removed by washing with warm PBS. Next, the adherent cells were cultured at 37^0C at 5% CO_2 in air for 48 h in 1 ml RPMI 1640, containing 10% FCS, 50 µg streptomycin and 50U penicillin and 10 µg/ml LPS in the presence (test group) or absence (control group) of different concentrations of *Ganoderma lucidum* (JHS natural products- Reishi extract with 12%polysaccharide and 6% triterpenes- USA) ranging from 5 to 160 µg/ml. Culture medium was subsequently removed from each well, placed into 1.5ml plastic micro tubes and centrifuged at 13000g for 10 minutes at room temperature. Finally, the supernatants from the centrifuged tubes were transferred to clean tubes and were stored at -70^0C until they were analysed for cytokines using ELISA kits (Bender Med System Company, USA).

Nitric Oxide Assay: Nitric oxide (NO) is an unstable product and difficult to detect and is easily converted to nitrite (NO2-) and nitrate (NO3-), so it is measured as NO2. Nitrite concentration in the supernatant was measured using a microplate assay method as described earlier (15). Briefly 50 µl aliquot was mixed with an equal volume of Griess reagent [(1% sulphanilamide, 0.1% naphtylenediamine dihydrochloride and 2.5% H3PO4) (Sigma Chemical Co)] and incubated at room temperature for 10 minutes to form a chromophore. After 10 minutes absorbance was measured at 540 nm in a Multiscan Titertek MCC/340. NO2- was determined using sodium nitrite (NaNO2) as standard. The concentration of NO2- obtained in the experiments was corrected by subtracting NO2- present in fresh medium alone.

Statistical Analysis. Data expressed as the mean ± SD. An analysis of variance (ANOVA) was used to determine differences between the control and test wells. When statistically significant differences ($p<0.05$) were found between the groups, unpaired t-test was used to determine the level of significant difference between the control and the test group.

RESULTS

The results showed that G. lucidum at concentrations from 5 to 40µg/ml induced cell death between 5-10% which was not significantly different from the control group (Figure1). The results of 48h cell culture incubation showed that IL-1β, IL-12, TNF-α, and NO production by peritoneal macrophages treated with 5-160 µg/ml G. lucidum was increased in a dose dependent manner (Figures 2,3,4 and Table 1) while for IL-12 the maximum and minimum release was induced in response to 5µg/ml (44.44% and $p<0.001$), and 160 µg/ml (8.3% and $p<0.005$) G. lucidum respectively, compared to the control group (Figure 3, and Table 1). On the other hand, the results showed that minimum enhancement in TNF-α release was induced in response to concentrations of 5, 10 and 20 µg and the increase was not significantly different from the control group. The highest enhancement of TNF-α was induced in response to 160 µg/ml G. lucidum (75% increase, $p<0.001$; Figure 4 and Table 1). Nitric Oxide production by peritoneal macrophages was also increased in response to different concentrations of G. lucidum in a dose dependent manner (Figure 5).

Table1. The percentage of increased of IL-1β, IL-12, TNF-α and NO release by mice peritoneal macrophages after treatment with different concentrations of G. lucidum compared with control group

Different concentrations of G. lucidum	IL-1β	IL-12	TNF-α	NO
5 µg	6.66	*44.44	1.25	2.4
10 µg	****13.33	*33.3	5	4
20 µg	***20	*25.5	2.5	*60
40 µg	*40	*20	****8.75	*58.4
80 µg	*73.33	*16.6	*43.75	*60
160 µg	*106.66	**8.3	*75	*170.4

Significant * P<001, **<0.005 ***P<0.01, ****P<0.05

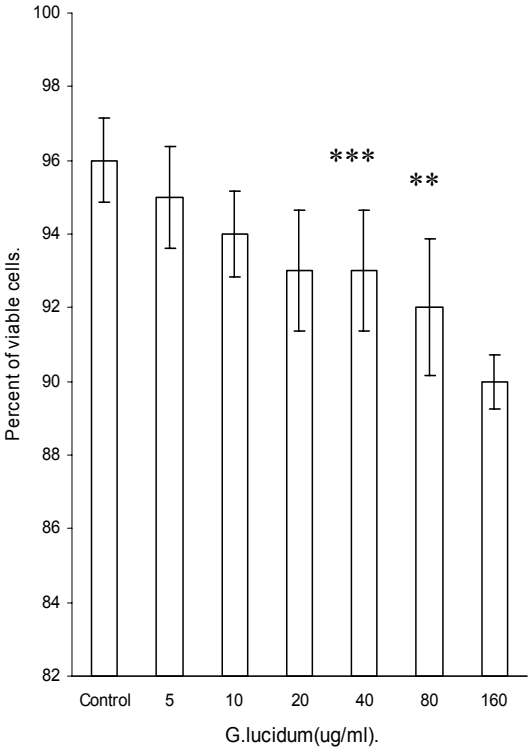

Figure1. Effect of different concentrations of G.lucidum on viability of mice peritoneal cells. Peritoneal cells were extracted as described in material and method, plated out and incubated for 48h in the presence or absence of different concentrations of G.lucidum. Cell viability was checked by trypan blue exclusion test.(***p<0.025, **p<0.005). Cell viability= (number of viable cells)/(sum of viable and dead cells) X 100.

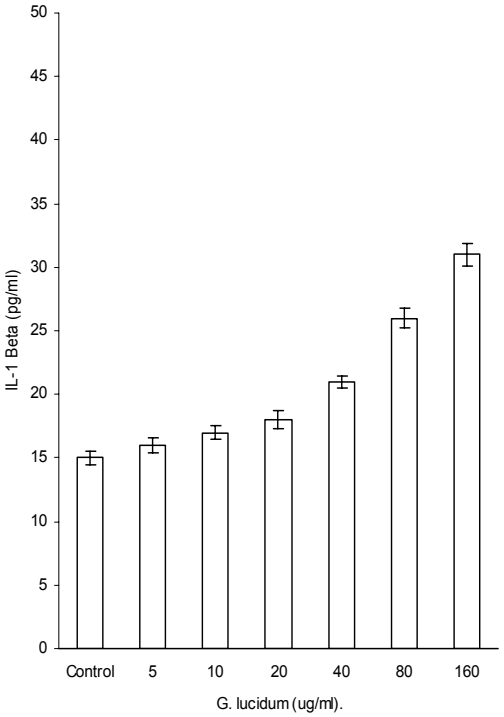

Figure 2. Effect of different concentrations of G. lucidum on IL-1 β production by mice peritoneal macrophages. Peritoneal macrophages were extracted as described in material and method, plated out and incubated for 48h in the presence or absence of different concentrations of G. lucidum. IL-1β was measured by ELISA kit.

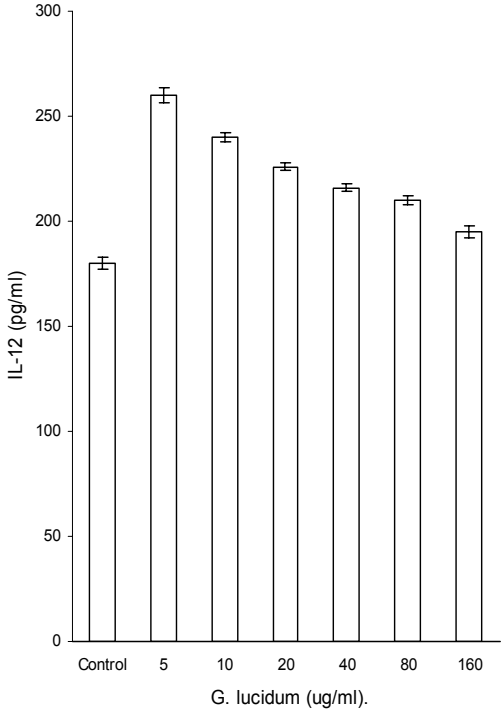

Figure 3. Effect of different concentrations of G. lucidum on IL-12 production by mice peritoneal macrophages. Peritoneal macrophages were extracted as described in material and method, plated out and incubated for 48h in the presence or absence of different concentrations of G. lucidum. IL-12 was measured by ELISA kit.

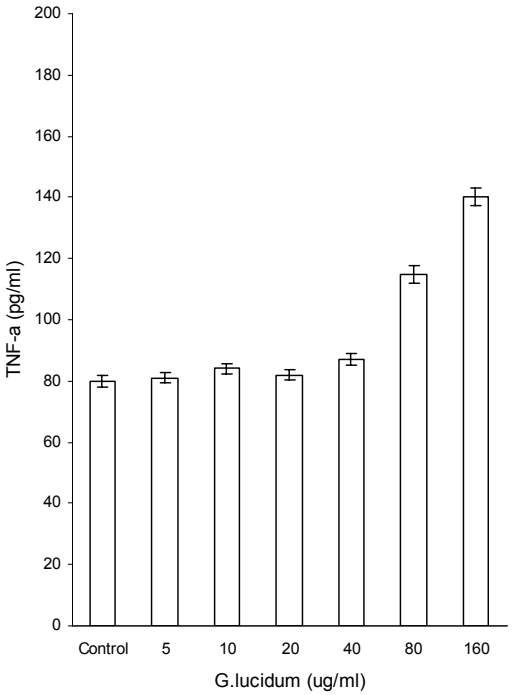

Figure4. Effect of different concentrations of G. lucidum on TNF-α production by mice peritoneal macrophages. Peritoneal macrophages were extracted as described in material and method, plated out and incubated for 48h in the presence or absence of different concentrations of G. lucidum. TNF-α was measured by ELISA kit.

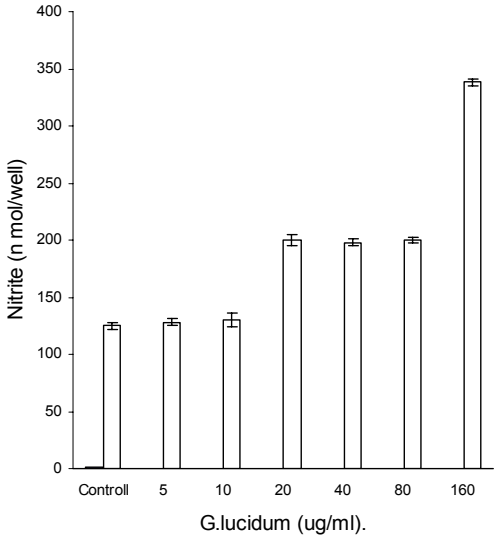

Figure 5. Effect of different concentrations of G. lucidum on NO production by mice peritoneal macrophages. Peritoneal macrophages were extracted as described in material and method, plated out and incubated for 48h in the presence or absence of different concentrations of G. lucidum. NO was measured by Gries method.

DISCUSSION

In the present study, we determined the efficacy of low doses of G. lucidum in cytokine production by mice peritoneal macrophages. It was found that G. lucidum at concentrations of 5-160 µg/ml enhanced IL-1β release by peritoneal macrophages. Results of IL-1β are in agreement with the findings of others reporting an increase of IL-1, IL-6, TNF-α and IFN-γ by human macrophages and T-lymphocytes after incubation with polysaccharides from fresh fruiting bodies of G. lucidum (16,17). In support of the above mentioned effect of G. lucidum, previous studies by other investigators have shown that G. lucidum can activate the function of macrophages, T lymphocytes and NK cells and induces cytokines such as TNF-α, interleukins and IFNs in human and mice immune cells (16,18,19). The enhancement of IL-1β production in this study is also supported by the findings of Chen et al. (20), who reported that F3, a fraction of G. lucidum activates the expression of IL-1, IL-6, IL-12, and TNF-α in mouse splenocytes.
Increased level of TNF-α in our experiments is in agreement with the results reported by Berovic (21). In our study G. lucidum was added to the microenvironment of mice peritoneal macrophages in tissue culture, however Berovic, used human peripheral blood mononuclear cells. In contrast to above mentioned results, a decrease in plasma concentration of IL-1 and TNF-α in colorectal cancer patients after treatment with G. lucidum was reported (5).
We also showed that G. lucidum at concentrations used enhanced IL-12 production. However, in contrast to IL-1β and TNF-α, the highest level of IL-12 released was in response to 5 µg of G. lucidum which was 44.4% more than the control group ($p<0.001$). The lowest level of IL-12 production was induced in response to 160 µg G. lucidum. Therefore, it seems that G. lucidum at above mentioned concentrations could stimulate macrophages in terms of IL-12 production and modulate Th cells through Th1 cells and the production of IL-2 and IFN-γ. Supporting this view, another study (10), showed that the oral administration of 500 mg/kg of G. lucidum resulted in a significant increase in IFN-γ production. It was also demonstrated that stimulation of splenic adherent cells from these mice with LPS resulted in a significant increase in IL-12 production (10). These results are in favour of a study (22) indicating that the mouse spleen cells treated with G. lucidum induce more cytokine production especially IL-1, IL-2 and IFN-γ compared with control group. Supporting this view an enhancement of phagocytic activity and an increase in IL-1 production in peritoneal macrophages of mice after an oral administration of G. lucidum was reported (23).
The increased levels of IL-1β and IL-12 correspond to the amount of NO produced by peritoneal macrophages, where NO production was increased in response to G. lucidum in a dose dependent manner. It has previously been reported that IL-1β and TNF-α are potent stimulators of NO production by peritoneal macrophages (24)**.** In this study, it was found that NO, IL-1β, and TNF-α release were increased in response to G. lucidum. This indicates that G. lucidum may indirectly stimulate NO production by peritoneal macrophages through the enhancement of IL-1β and TNF-α release. In contrast with our finding, an inhibitory effect of G. lucidum on LPS-induced NO production by RAW 264.7 macrophages was reported earlier (6). However, the differences in the results could be due to the fact that in our study we used mice peritoneal macrophages which is different from RAW 264.7 cell line. Moreover in that study LPS-IFNγ activated macrophages treated with G.lucidum were used without a control group (6). It is concluded

that *Ganoderma lucidum* may improve immune responses in mice peritoneal macrophages through enhancement of cytokine release.

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